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INHIBITION OF BLEOMYCIN-INDUCED CELLULAR DNA STRAND SCISSION BY 1,10-PHENANTHROLINE

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Abstract—Inhibition by 1,10-phenanthroline of cellular DNA strand scission induced by the antitumor antibiotic bleomycin in Ehrlich ascites tumor cells was studied. DNA alkaline elution was performed on cells after 1-hr bleomycin treatments. Pretreatment for 24 hr with initial 1,10-phenanthroline concentrations of 0.2 nmol/10^5 cells, which depletes cells of ferritin iron by 80%, had no consistent effect on bleomycin strand breakage. However, simultaneous treatment with 3.1 nmol of 1,10-phenanthroline/10^5 cells and with bleomycin concentrations from 5 to 25 μM decreased both apparent double-stranded breaks and random breakage. When cells were treated with both 3.1 nmol of 1,10-phenanthroline/10^5 cells and 25 μM bleomycin, washed free of both drugs, and incubated at 37° for 1 hr, the resulting breakage was equivalent to that found in cells treated with bleomycin only. When the combination treatment was extended to 4 hr, cell washing and reincubation resulted in increased strand scission, as compared with strand scission in cells treated with bleomycin only. Growth inhibition by bleomycin was not affected appreciably by temporary suppression of DNA strand breakage activity.

DNA breakage by bleomycin is believed to be responsible for the cytotoxic effect of the drug [1]. Cell-free DNA degradation, modeled in a variety of ways *in vitro*, is greatly stimulated by the addition of iron [2–5], and the active form of the drug has been attributed to an iron–bleomycin complex [1]. However, under certain conditions other metals such as copper, cobalt, and manganese can also support bleomycin-dependent DNA breakage [6–9]. Additionally, treatment of cells with iron [Fe(III)], copper, and zinc complexes of the drug results in degrees of inhibition of cell proliferation similar to those of the metal-free drug [10, 11]. Intracellular metal exchange reactions in which bleomycin acquires iron, thus enabling DNA damage reactions, have been proposed to account for the similar toxicities of metal-free bleomycin and copper bleomycin [12, 13]. In one study of this hypothesis, experiments with iron(III)bleomycin, copper(II)-bleomycin, and the metal-free form demonstrated no differences in the degree of DNA degradation [14]. The results suggest that either intracellular metal exchange reactions occur rapidly, which convert various metallobleomycins to a common, active form, or different metallobleomycins can act independently as cytotoxic agents.

An alternative approach taken to examine the requirement for metals in the cellular mechanism of bleomycin has been to treat cells with metal-chelating agents, which, in principle, can compete with the drug for available intracellular metals, particularly iron. If reductions in DNA damage or cytotoxicity are noted, then it is inferred that the chelating agent has successfully withheld the critical metal ion from the drug. For example, male rat germ cells have been reported to show decreased bleomycin-induced DNA breakage after pretreatment with desferrioxamine, a chelator with high specificity for iron(III) [15]. Nonetheless, desferrioxamine does not reduce bleomycin toxicity in V79 cells [16].

Another reagent which has been used is 1,10-phenanthroline, which forms a stable complex with Fe(II) as well as other transition metal ions. It has been shown, for example, that 1,10-phenanthroline inhibits H_2O_2-induced cellular DNA damage and toxicity [17, 18]. From this result it has been argued that intracellular iron is needed to catalyze the Fenton reaction, reducing H_2O_2 to the reactive hydroxyl radical, and that 1,10-phenanthroline intervenes to chelate iron and inhibit this chemistry. With bleomycin, 1,10-phenanthroline was found to cause a small reduction of the long-term growth inhibitory effect of the drug in a select combination of bleomycin and 1,10-phenanthroline concentrations [19]. Other work showed that 1,10-phenanthroline could achieve modest inhibition of DNA degradation and largely prevent formation of chromosomal aberrations by bleomycin in Chinese hamster ovary cells [20]. In contrast, though a low concentration of 1,10-phenanthroline reduces the iron and zinc content of Ehrlich ascites tumor cells without any apparent detrimental effect on growth, no differences were found in growth inhibition caused by bleomycin in cells pretreated or concurrently exposed to this reagent, in comparison with cells incubated with bleomycin only [21].

As an extension of this work, we have investigated features of the inhibition of bleomycin-induced cellular DNA damage by 1,10-phenanthroline. The results indicate that production of DNA strand breaks is inhibited by 1,10-phenanthroline, but that in order to observe this inhibition, levels of 1,10-phenanthroline must be present which are toxic to cells during long-term treatments. This effect of 1,10-phenanthroline is reversible, for DNA damage attributable to residual bleomycin activity was observed after cells were washed free of extracellular bleomycin and 1,10-phenanthroline.

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Table 1. Effect of 24 hr 1,10-phenanthroline pretreatment on the size of the F1 fraction

Treatment	Bleomycin concentration (μM)	Size of F1 fraction		
		Expt. 1	Expt. 2	Expt. 3
None	0	0.01	0.03	0.02
1,10-Phenanthroline	0	0.01	0.01	0.01
Bleomycin	5	0.17	0.26	0.14
1,10-Phenanthroline + bleomycin	5	0.09	0.18	0.10
Bleomycin	10	0.15	0.11	0.11
1,10-Phenanthroline + bleomycin	10	0.10	0.19	0.14
Bleomycin	25		0.15	0.21
1,10-Phenanthroline + bleomycin	25		0.11	0.13

Cells were treated with or without 1,10-phenanthroline (0.2 nmol/10^5 cells) for 24 hr prior to, and during, bleomycin treatments (1 hr).

MATERIALS AND METHODS

All solutions were prepared using doubly-distilled deionized water and acid- or NaOH/EDTA-washed glassware. Blenoxane was the gift of Bristol Laboratories, Syracuse, NY. Bleomycin concentrations were quantitated by means of the ε_{290} of 14,000 $M^{-1}\,cm^{-1}$ obtained for blenoxane [22]. Labeled thymidine was obtained from RPI Products, Mount Prospect, IL. 1,10-Phenanthroline was obtained from the Aldrich Chemical Co., Milwaukee, WI.

Ehrlich ascites tumor cell suspensions were grown in Eagle's Minimal Essential Medium (Gibco, Grand Island, NY) containing Earle's salts (MEM*) and 1 or 2.5% fetal bovine serum at 37°. Cultures were resuspended daily in fresh medium. Over a 24-hr period, these cells grew by factors of about 2.2 (1% serum) or 2.9 (2.5% serum), with no difference in viability. Cell viability was assessed by trypan blue exclusion.

Alkaline elution. The methodology for DNA strand scission analysis of bleomycin-treated cells using Co^{2+} to suppress adventitious strand scission activity has been described [14]. Briefly, the strictly deproteinizing alkaline elution method of Kohn *et al.* [procedure B of Ref. 23] was modified to include a pre-cell lysis wash of cells and filter (2 μM polycarbonate, Nucleopore Corp., Fullerton, CA) with phosphate-buffered saline (PBS) containing 0.5 mM $CoCl_2$ (CoPBS), followed by washing with non-cobalt-containing PBS. An internal standard of 300-rad γ-irradiated, [^{3}H]dThd-labeled cells was also used to provide a corrected time scale of elution from each filter for comparison purposes. An increased slope of elution (higher negative value) indicates an increased degree of strand breakage [14].

One to two hours prior to treatment, [2-^{14}C]dThd-labeled cells were suspended in fresh growth medium at 8×10^5/mL. Cells already growing in phenanthroline (initial concentration ratio, 0.2 nmol/10^5 cells) were resuspended in medium containing a similar concentration ratio of 1,10-phenanthroline prior to addition of bleomycin. For simultaneous treatment with bleomycin and high levels of 1,10-phenanthroline (3.1 nmol/10^5 cells, about 25 μM), 1,10-phenanthroline was added 5 min prior to addition of bleomycin. Under the conditions utilized in this report, cells treated with 1,10-phenanthroline, bleomycin, or a combination of the two resist uptake of trypan blue for at least 6 hr [11, †].

Following treatment, an aliquot of treatment medium containing 5×10^5 cells was added to 15 mL of ice-cold CoPBS. After addition of 5×10^5 internal standard cells, cells were added to elution barrels and washed with an additional 50 mL CoPBS and 50 mL non-cobalt-containing PBS. When the level of the last wash reached the neck between upper and lower chambers of the Swinnex funnel [23], 5 mL of lysis solution (2% SDS plus 25 mM EDTA, pH 10) was added to the upper chamber and allowed to flow through the filter until the neck was reached. An additional 2 mL of lysis solution containing proteinase K was added and again allowed to flow through until the neck was reached. This second lysis step was allowed to rest undisturbed for 40 min, after which eluting solution was added to the upper chamber and the pump started. The total lysis volume collected (7 mL) was counted for radioactivity and the ^{14}C activity is referred to as Fl.

The DNA was eluted with tetrapropylammonium hydroxide, pH 12.1, containing 0.1% SDS and 25 mM EDTA. Forty-minute (1.4-mL) fractions were collected and analyzed for radioactivity after addition of Ecolite Liquid Scintillation Fluid (ICN Biomedicals, Irvine, CA) containing 0.7% acetic acid. Elution graphs indicate the fraction of total cellular DNA eluting in the lysis solution (F1) on the ordinate. The slopes of elution curves in the range of internal standard retentions 0.8 to 0.4 were obtained by least squares analysis [14].

RESULTS

Growth of Ehrlich ascites tumor cells in the presence of 0.2 nmol of 1,10-phenanthroline/10^5 cells initial ratio is unaffected; yet cytosolic ferritin (high molecular weight) iron stores are depleted [21]. Experiments were performed to examine whether cells pretreated with this concentration of

* Abbreviations: MEM, Eagle's Minimal Essential Medium plus Earle's salts; PBS, phosphate-buffered saline; CoPBS, phosphate-buffered saline plus 0.5 mM $CoCl_2$; F1, fraction of total cell DNA eluting in lysate of alkaline elution experiment; and SDS, sodium dodecyl sulfate.

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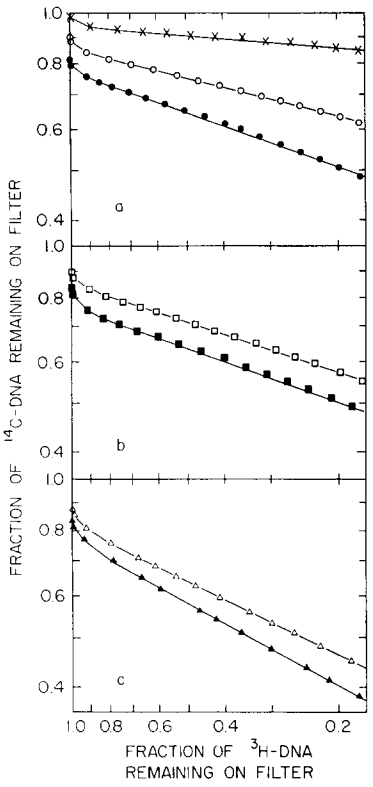

Fig. 1. Absence of effect of continuous growth in 0.2 nmol of 1,10-phenanthroline/10^5 Ehrlich cells on alkaline elution of DNA after bleomycin treatment. Cells were grown in 1% fetal bovine serum and bleomycin treatments were for 1 hr. Data are plotted against elution of internal standard [^{3}H]DNA from 300-rad γ-irradiated cells and includes the fraction of treated cell [^{14}C]DNA contained in the lysate (F1) on the ordinate. Although in this experiment F1 for each bleomycin concentration appeared decreased by 1,10-phenanthroline treatment, this was not observed in all experiments (Table 1). Symbols: (a) ×, control; ●, 5 μM bleomycin; and ○, 5 μM bleomycin plus phenanthroline; (b) ■, 10 μM bleomycin; and □, 10 μM bleomycin plus phenanthroline; and (c) ▲, 25 μM bleomycin; and △, 25 μM bleomycin plus phenanthroline.

1,10-phenanthroline exhibit altered DNA strand scission upon treatment with bleomycin. Cells were grown in 0.02 to 0.03 μCi/mL [^{14}C]dThd for 24 hr. The growing culture was then divided into two spinner bottles, one of which contained 0.2 nmol of 1,10-phenanthroline/10^5 cells. Twenty-four hours later, cells from both cultures were treated with bleomycin and analyzed by DNA alkaline elution. No consistent pattern of inhibition by 1,10-phenanthroline was observed in either the fraction of treated cell DNA appearing in the lysis solutions prior to elution (Table 1), or in the rate of alkaline elution of DNA. Elution curves from a representative experiment are plotted in Fig. 1.

In contrast to results with low concentrations of 1,10-phenanthroline, concurrent treatment of cells with higher levels of 1,10-phenanthroline (3.1 nmol/10^5 cells) and bleomycin (5–25 μM) was found effective in considerably reducing DNA degradation by bleomycin during 1-hr treatments (Fig. 2). The inhibition was apparent in both decreased lysis radioactivity levels and in decreased slope of curves during alkaline elution of DNA. At these levels of 1,10-phenanthroline, there was sometimes an effect on the elution of DNA from 1,10-phenanthroline-treated control cells in the absence of bleomycin. This was limited to a small increase in the rate of elution, usually in the initially eluting fractions; no increase was evident in DNA levels in lysis fractions. While not present in this experiment (not shown), minor levels of DNA damage resulting from 1,10-phenanthroline treatment were more prominent in the experiments of Figs. 4 and 5.

The higher concentration of 1,10-phenanthroline was itself toxic to cells, leading to a reduction in viable cells in suspension cultures by 28% after 24 hr of continuous incubation. For this reason it was not possible to assess the role of bleomycin-induced DNA strand scission in causing the growth-inhibitory effects of the drug over a 24-hr period by using 1,10-phenanthroline to prevent DNA damage. However, short-term treatment of Ehrlich cells with bleomycin (30–60 min) results in nearly as much growth inhibition after 24–48 hr as continuous treatment with drug over this period [11]. Thus, to examine whether DNA damage produced during initial treatments may be responsible for long-term growth inhibition, an experiment was conducted to examine whether the growth inhibitory effect of 1- to 6-hr bleomycin treatments can be altered by concomitant 1,10-phenanthroline exposure which inhibits DNA damage during this time period. Cells were treated with 1,10-phenanthroline, bleomycin, or combined phenanthroline plus bleomycin, followed by washing and resuspension in fresh medium in the absence of added bleomycin and 1,10-phenanthroline. Cells were counted at the end of 24 hr after the start of drug treatment. The results, shown in Fig. 3, indicate that there was little effect of the simultaneous presence of 1,10-phenanthroline on reducing growth inhibition produced by initial 1-hr treatments. Additionally, simultaneous treatment with 1,10-phenanthroline for up to 6 hr did not lead to a marked difference in growth inhibition from that obtained with bleomycin only.

Intracellular bleomycin, once taken up, does not efflux from Ehrlich cells [11], whereas 1,10-phenanthroline, a relatively non-polar molecule, may be expected to diffuse from cells once the external concentration of 1,10-phenanthroline is reduced. Thus, an experiment was performed to see if the effect of 1,10-phenanthroline on bleomycin-induced DNA strand scission was reversible. DNA degradation in Ehrlich cells was analyzed 1 hr after cells were washed free of bleomycin and 1,10-phenanthroline subsequent to a 1-hr treatment with the two reagents. The results, shown in Fig. 4, indicate that the initial inhibition of bleomycin activity by 1,10-phenanthroline was removed by washing of cells, for 1 hr later the DNA damage caused by residual bleomycin was equivalent to that from a 1-hr treatment of cells with drug. Control treatments with phenanthroline present continuously during 1- and 2-hr treatments with bleomycin showed continued inhibition of strand scission.

A final experiment was conducted to examine whether continued inhibition of strand scission

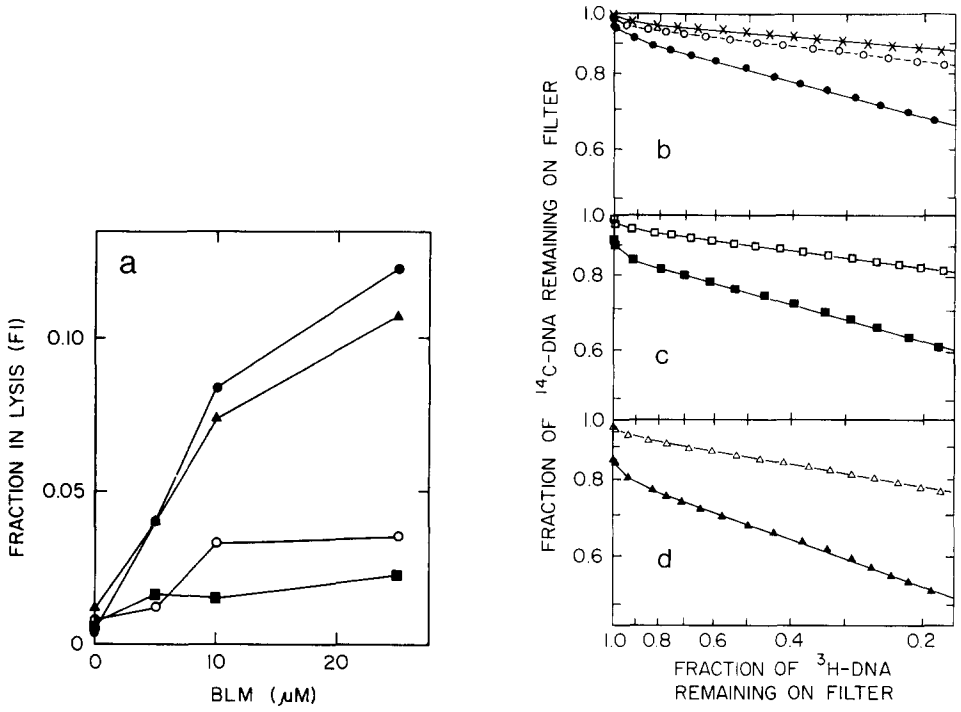

Fig. 2. Effect of simultaneous treatment of Ehrlich cells with 3.1 nmol of 1,10-phenanthroline/10^5 cells on DNA degradation by bleomycin. Cells were grown in 2.5% serum. (a) F1 fraction in two experiments; (b) through (d), alkaline elution curves from a representative experiment. Symbols in panel (a) represent F1 fraction in the absence of phenanthroline in two experiments (● and ▲) and corresponding F1 fraction in the presence of phenanthroline (○ and ■ respectively). Symbols in (b) represent ×, control; ●, 5 μM bleomycin; and ○, 5 μM bleomycin plus phenanthroline; (c) ■, 10 μM bleomycin; and □, 10 μM bleomycin plus phenanthroline; (d) ▲, 25 μM bleomycin; and △, 25 μM bleomycin plus phenanthroline. Treatments were for 1 hr.

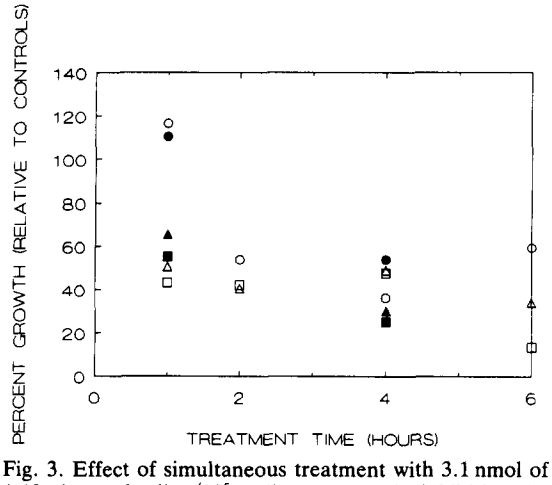

Fig. 3. Effect of simultaneous treatment with 3.1 nmol of 1,10-phenanthroline/10^5 cells on growth inhibition by bleomycin. Cells adapted to 1% serum were treated for the indicated times, washed once in MEM, and resuspended in complete medium at 6–8 × 10^5/mL. Viable cells were counted on a hemocytometer after staining with trypan blue 24 hr after the start of treatment and normalized with respect to initial cell counts for each treatment. Open symbols (○, □, △) represent results of one experiment for each treatment period, while filled symbols (●, ■, ▲) represent results of a second experiment. Symbols: 1,10-phenanthroline (○, ●), 25 μM bleomycin (□, ■), or combined 1,10-phenanthroline plus bleomycin (△, ▲).

activity of bleomycin remained during 4 hr of simultaneous incubation with 1,10-phenanthroline and to examine whether strand scission activity returned after washing and subsequent reincubation of cells in fresh medium (Fig. 5). In repeated experiments, continued suppression of DNA damage was found after 4 hr of combined treatment. This inhibition appeared somewhat diminished due to increased breakage resulting from longer incubations with 1,10-phenanthroline. Strand scission after 1 hr of recovery from the combined treatment (in the absence of both drug and 1,10-phenanthroline in the external medium) was enhanced by comparison with 4-hr treatments with bleomycin or 1,10-phenanthroline only. Both F1 and the slopes of elution were increased to a degree which is greater than the sum of corresponding components for individual bleomycin and 1,10-phenanthroline treatments (4 hr plus 1 hr recovery) (calculations not shown). Notably, both 1 hr and 4-hr bleomycin controls produced equivalent or nearly equivalent strand scission, indicating that the balance between DNA damage and repair processes observed after 1 hr was hardly altered by longer incubation of cells with the drug.

DISCUSSION

The antiproliferative action of bleomycin is believed due to its degradation of cellular DNA [1]. Direct products of bleomycin action on cell-free

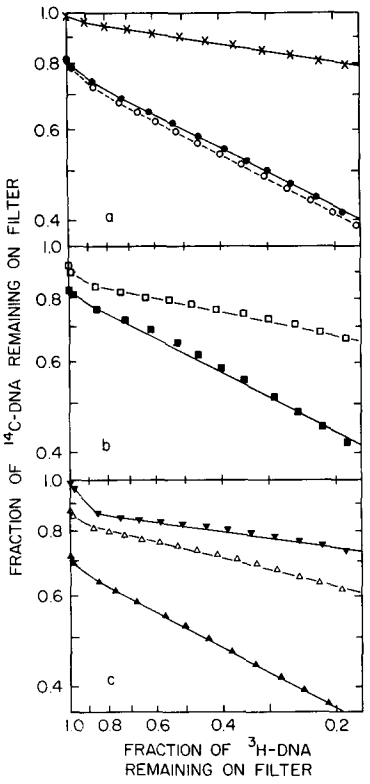

Fig. 4. Effect of washing cells free of bleomycin and 1,10-phenanthroline on extent of DNA strand scission after 1 hr of treatment. Cells growing in 1% serum were treated with 25 μM bleomycin, 3.1 nmol of 1,10-phenanthroline/10^5 cells, or both bleomycin and 1,10-phenanthroline. In (a), cells were treated with bleomycin (●) or bleomycin plus phenanthroline (○) for 1 hr, followed by washing cells and growth in fresh medium for 1 hr. In (b), cells were treated with bleomycin (■) or bleomycin plus phenanthroline (□) for 1 hr with no post-treatment wash; and in (c), cells were treated as in (b) for 2 hr (bleomycin only, ▲; bleomycin plus phenanthroline, △). Also shown are the elution of untreated controls (×, panel a), and the elution of cells treated with phenanthroline for 2 hr (▼, panel c).

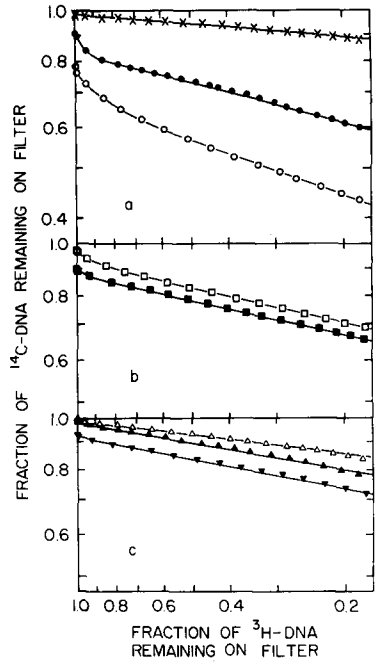

Fig. 5. Effect of washing cells free of bleomycin and 1,10-phenanthroline on extent of DNA strand scission after 4 hr of treatment. Cells growing in 1% serum were treated with 25 μM bleomycin, 3.1 nmol of 1,10-phenanthroline/10^5 cells, or both bleomycin and 1,10-phenanthroline. In (a), cells were treated with bleomycin (●) or bleomycin plus phenanthroline (○) for 4 hr, followed by washing cells and growth in fresh medium for 1 hr. In (b), cells were treated with bleomycin (■) or bleomycin plus phenanthroline (□) for 4 hr with no post-treatment wash; and in (c), cells were treated with phenanthroline for 4 hr (▲) or for 4 hr followed by washing and resuspension in fresh medium for 1 hr (△). Also shown are the elution of untreated controls (×, panel a) and the elution of cells treated with bleomycin for 1 hr (▼, panel c).

DNA are single- and double-strand breaks, free bases, and base-propenals [2–5, 24]. Levels of these products are dependent upon the presence of metals, particularly iron. However, a role for direct involvement of iron in the DNA damage induced by bleomycin in cells has not been demonstrated, in part because of the difficulties in obtaining living systems sufficiently devoid of this trace element [25].

The bidentate chelating agent, 1,10-phenanthroline, forms a 1:3 complex with Fe^{2+}, with a log stability constant of 21 at pH 7 [21, 26]. The stability constant of Fe(II)bleomycin at this pH is less than 9.7 [25]. As a consequence, 1,10-phenanthroline rapidly and completely extracts Fe(II) from iron bleomycin under stoichiometric conditions [21]. Thus, because 1,10-phenanthroline concentrates in Ehrlich cells to much higher levels than bleomycin ([21, 27] and discussed below), this reagent may be a strong competitor with bleomycin for cellular iron, leading to inhibition of formation of DNA damage by the drug.

While chelating agents have been used extensively to probe the behavior of metals in cells, it is generally not easy to identify whether, or how, they perturb metal-dependent reactions in cells, for they may form complexes with several biologically essential metals and exert other effects independent of their metal chelation properties. For example, 1,10-phenanthroline decreases or alters the intracellular distribution of zinc as well as iron in Ehrlich cells [21]. Researchers interested in zinc metabolism have ascribed the inhibition of cell proliferation caused by 1,10-phenanthroline to a disruption of zinc, not iron, metabolism [27]. Others have suggested that a copper–phenanthroline complex may be responsible for the growth-inhibitory properties of this metal-chelating agent [28]. Further, 1,10-phenanthroline may exert effects distinct from its ability to bind metals, such as direct binding to DNA, which may interfere with DNA-oriented activities. Thus, it is difficult to ascribe the impact of 1,10-phenanthroline on bleomycin activity only to its interaction with species of cellular iron [21]. Nevertheless, competition for cellular metals, one of which may be iron, by 1,10-phenanthroline has been proposed as the basis of the inhibition of DNA synthesis [19], formation of chromosome aberrations [20], and damage leading to increased DNA alkaline unwinding

[20] induced by bleomycin. With this background in mind, we have examined some of the features of 1,10-phenanthroline inhibition of bleomycin strand scission in Ehrlich cells. The results show that 1,10-phenanthroline provides a useful tool for control of the strand scission activity of the drug.

A previous study showed that pretreatment of Ehrlich cells with 1,10-phenanthroline largely depletes cytosolic ferritin of iron and also reduces cellular zinc [21]. Yet under these conditions the ligand had no effect on DNA strand scission caused by bleomycin (Table 1; Fig. 1). Growth inhibition by bleomycin also remains unaffected [21]. Thus, if iron is required for these processes, the drug can still acquire it when the major storage form, ferritin, has lost most of its supply of the metal.

Notwithstanding these results at low concentrations, short-term treatments with higher levels of 1,10-phenanthroline were effective in inhibiting bleomycin strand scission (Fig. 2). Interestingly, 1,10-phenanthroline decreased both the fraction of treated cell DNA appearing in the lysis solution (F1) as well as the rate of elution. F1 is thought to result primarily from double-strand breakage, while the latter phase of elution, occurring under denaturing conditions, is believed to result from random, single-strand breakage [14]. For both types of damage, the inhibition by 1,10-phenanthroline was nearly complete, suggesting that they arise from processes which share chemical events that are sensitive to this reagent.

The levels of breakage measured after bleomycin treatments reflect a balance between rates of breakage induction and rates of repair [29, 30]. The reduced level of breakage found in cells during concurrent treatment with 3.1 nmol 1,10-phenanthroline/10^5 cells and 25 μM bleomycin (Fig. 2) may be due to either inhibition of breakage by 1,10-phenanthroline, a stimulation of repair processes, or some combination of both processes. Levels of breakage returned to those of bleomycin-treated cells exposed in the absence of 1,10-phenanthroline within 1 hr after removal of extracellular phenanthroline and bleomycin (Fig. 4). These results suggest that after thorough washing, 1,10-phenanthroline is lost from cells, but functionally active bleomycin remains. Its action on DNA establishes a steady state between strand break induction and DNA repair processes as rapidly as if cells were treated with bleomycin alone for 1 hr.

The reversible suppression of the adverse effects of bleomycin on DNA by 1,10-phenanthroline was observed for as long as 4 hr (Fig. 5). Two features of these experiments deserve mention. First, the DNA damage incurred by 4-hr incubation of cells with bleomycin was essentially the same as that seen after 1 hr. Thus, a steady state betwen DNA damage and repair exists for a number of hours after the initial 1-hr treatment. Second, breakage after treatment with both 1,10-phenanthroline and bleomycin for 4 hr followed by 1 hr of recovery (Fig. 5) was greater than that seen with bleomycin alone incubated continuously with cells for this period.

The basis of the latter effect is not known with certainty. One explanation is that 1,10-phenanthroline may block cells in a phase of the cell cycle [31]. When the block is removed by washing, the cells may progress into a phase in which the levels of strand scission are expressed to a greater extent [32].

In control treatments, low levels of strand scission were seen after addition of 1,10-phenanthroline alone (Figs. 3 and 5). Cellular DNA breakage by 1,10-phenanthroline has not been observed by other investigators using shorter treatment times and less sensitive methods [17, 18, 20], though breakage of DNA in isolated nuclei has been observed [20]. The source of this phenomenon was attributed to formation of a copper–phenanthroline complex and action on DNA through a mechanism which has been characterized *in vitro* [18, 33]. The validity of this mechanism occurring in Ehrlich cells is considered elsewhere.* In the present work, it is sufficient to observe that there was no long-term effect on growth associated with the modest degree of DNA damage obtained after 1-hr treatments, though there was a dramatic decrease in growth following longer treatments (Fig. 3). Other investigators have also shown no effect on cell survival resulting from short treatments (< 1 hr) with 1,10-phenanthroline [17]. Growth inhibition by longer treatments (2–4 hr) with 1,10-phenanthroline was not accompanied by marked initial levels of DNA strand scission (Figs. 4 and 5).

For all treatment times, growth inhibition correlated with the persistence, or recovery, of strand scission after washing cells treated with bleomycin or bleomycin plus 1,10-phenanthroline. These observations indicate that residual bleomycin which remains in cells after washing can exert at least some of its long-term effects without diminution even though it carries out little DNA strand cleavage during the initial 1–4 hr of incubation with the cells. It also suggests that at least in Ehrlich cells, processes of inactivation of bleomycin such as that catalyzed by bleomycin hydrolase are not effective during the first 6 hr of exposure of cells to the drug. This is consistent with an apparent absence of metabolism of intracellular bleomycin by bleomycin hydrolase in other cultured cells [34, 35].

The retention of DNA damaging and growth inhibitory capacities after incubation with, and removal of, 1,10-phenanthroline is also consistent with previous demonstration that bleomycin is principally accumulated in a rapid process (40% of the 24-hr cumulative intracellular concentration in 60 min) followed by a much slower uptake reaction. Once associated with cells, it does not efflux into a drug-free medium [11].

Calculations were made of the relative degree of uptake of bleomycin and 1,10-phenanthroline in Ehrlich cells on the basis of previous studies [11, 27]. Over a 40-min incubation period, Ehrlich cells readily accumulate 21 and 28% of the non-metal form of 1,10-phenanthroline initially present in the extracellular medium at 8.5 and 4.3 nmol/10^5 cells respectively [27]. By extrapolation, at 3.1 nmol/10^5 cells, 29% uptake over a 60-min incubation could be expected, for a total uptake of 900 pmol/10^5 cells. Measurements with [^{3}H]bleomycin A_2 indicate about

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1 pmol/10^5 cells is accumulated during treatment at 40 nmol/10^5 cells for 1 hr [11]. Assuming proportionally less drug associates with cells exposed to 25 nmol/10^5 cells, the ratio of uptake of 1,10-phenanthroline to bleomycin is about 1400.

At present the mechanism of action of 1,10-phenanthroline cannot be attributed to its capacity to chelate iron. Still, the strong belief that iron bleomycin is the agent active in DNA damage caused by the drug [1] justifies some comments about the hypothesis that 1,10-phenanthroline exerts its effect through iron complexation. The demonstration that substantial depletion of ferritin iron by 1,10-phenanthroline or other means alters neither the inhibition of cell proliferation nor the DNA damage caused by bleomycin points to a direct competition between drug and 1,10-phenanthroline for iron as the key reaction [21]. This is further supported by an unpublished observation that 1,10-phenanthroline had little effect in depleting cellular iron during a short, 1-hr incubation with cells. It is also consonant with the facile reversibility of inhibition, which implies that 1,10-phenanthroline must be present in order to suppress bleomycin-induced DNA damage. The concentration ratio of intracellular, 1,10-phenanthroline to bleomycin of 1400 calculated above is more than enough to effectively remove all iron from bleomycin during *in vitro* experiments [21].

The ready reversibility of inhibition of strand cleavage also suggests that bleomycin can rapidly acquire iron for DNA breakage reactions as soon as 1,10-phenanthroline is diluted out of cells. When external 1,10-phenanthroline is removed from cell suspensions, some of the intracellular ligand must diffuse out of the cells, according to the current studies and those of others [17, 18, 31]. The volume of an Ehrlich cell is about 8×10^{-10} mL, based on the volume of a wet pellet after centrifugation. At 8×10^5 cells/mL, the volume occupied by Ehrlich cells represents 6×10^{-4} of the total treatment volume. Assuming rapid redistribution between interior and exterior of cells to equalize internal and external concentrations, only a small amount of 1,10-phenanthroline (0.9 pmol/10^5 cells) remains inside cells, which is approximately the same concentration of cell-bound bleomycin, and may be insufficient to compete stoichiometrically for iron from iron bleomycin. The proposal that 1,10-phenanthroline may block bleomycin strand scission activity by competition for cellular iron is thus consistent with known properties of 1,10-phenanthroline and bleomycin in Ehrlich cells.

Because 1,10-phenanthroline can interact with other metals in cells and may exert other, non-specific effects, this study does not exclude other possible explanations for our findings besides interaction of 1,10-phenanthroline with iron. Nevertheless, it does describe a novel, reversible means by which the DNA strand scission reactions of bleomycin in cells can be inhibited during short treatments without affecting long-term growth. These observations provide a basis for use of 1,10-phenanthroline in studies of the relationship of DNA damage to other processes which may accompany the presence of bleomycin in cells.

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