Potential of Bleomycin Cytotoxicity in *Saccharomyces cerevisiae*

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Lesions introduced into cellular DNAs prelabeled with [2-¹⁴C]thymidine or [6-³H]thymidine, as well as cell killing, were inhibited by the presence of EDTA during 20-min reactions of *Saccharomyces cerevisiae* cells with the low-molecular-weight bleomycin family of anticancer antibiotics. In contrast, the level of killing by low concentrations of bleomycin was higher among cells which had grown for three generations in defined synthetic complete medium supplemented with ferrous sulfate than among cells grown without iron supplementation. In *S. cerevisiae*, the uptake of iron is facilitated by a plasma membrane ferric reductase activity and a high-affinity ($K_m = 5 \times 10^{-6}$ M) ferrous uptake system. Lethal effects of 1.3×10^{-6} M bleomycin increased approximately 50% with 10^{-5} M Fe(II), nearly twofold with 10^{-4} M Fe(II), and 2.8 times with 10^{-3} M Fe(II). Thus, iron preloading is a new experimental approach to increasing and studying the effects of the glycopeptides on cellular DNAs and other cellular targets. This approach could also be used for studying and better understanding DNA repair genes and could serve as a model for studies of redox active chemicals in biological systems.

The requirement for divalent metal ions to form activated complexes of bleomycin with oxygen is satisfied by *Saccharomyces cerevisiae* cells, since exogenous supplements of Fe(II) are not required for introducing lesions into DNAs or for killing (22–28). On the basis of the requirement for Fe(II) for activity of bleomycin analogs in vitro (1–3, 6, 13, 23, 26, 32, 34, 39, 40, 43 [reviewed in references 4, 8, 12, 17–19, 29, 30, 33, 42, and 44]), we initiated studies many years ago with the working hypothesis that supplementing growing cells with iron could be an effective experimental approach to increasing and studying the biochemical reactivity and lethal effects of bleomycin. At the outset, it was anticipated that activities of bleomycin congeners might not be increased because of tight regulation of iron uptake by cells. It is now known that iron gets into *S. cerevisiae* readily, but the amount that gets in is regulated (7, 9, 20). The uptake of iron into *S. cerevisiae* is mediated by a plasma membrane ferric reductase activity and a high-affinity ($K_m = 5 \times 10^{-6}$ M) ferrous uptake system (7, 9, 20).

For the current report, experimental approaches were designed to mimic the cellular site of reaction of metal ions with the congeners and to serve as a model for studies of redox active chemicals in biological systems. We report here how the availability of ferrous iron in *S. cerevisiae* cells affects the activities of bleomycins.

MATERIALS AND METHODS

Culturing conditions, strains, and radioisotopes. Precultures of haploid strains A364A and H288 (14–16, 21, 22) were routinely grown at 23°C with aeration to a particular growth phase and cell titer in nonsynthetic complete medium (1% Bacto-yeast extract, 2% Bacto Peptone, 2% dextrose, 0.002% adenine sulfate) the day before radiolabeling to ensure an adequate supply of fresh cells. DNA and RNA in growing yeast cells were radiolabeled approximately 14 h from starting

inocula of 5×10^6 to 8×10^6 cells per ml in supplemented synthetic minimal medium (11, 21, 31) containing, per liter (wt/vol), 0.67% yeast nitrogen base without amino acids and ammonium sulfate, 0.005% ammonium sulfate, 1% succinic acid, 0.6% NaOH, 2% dextrose, 0.002% adenine, 0.003% isoleucine, 0.015% valine, and 0.002% tryptophan. [6-³H]uracil (New England Nuclear, Boston, Mass.; specific activity, 20 to 30 Ci/mmol) was added to 7 μ Ci/ml, or [2-¹⁴C]uracil (New England Nuclear; specific activity, 40 to 60 Ci/mmol) was added to 5 μ Ci/ml. A nonspecific label was used for nucleic acids because yeast cells lack the enzyme thymidine kinase, which is required for utilization of exogenous thymidine for incorporation into DNA. In later steps, RNA was selectively and completely hydrolyzed. Cells were harvested from the medium containing [6-³H]uracil or [2-¹⁴C]uracil by centrifugation at 4°C in an RC5B Sorvall SS34 rotor at $3,000 \times g$, washed once in deionized water, chased for 90 min in supplemented synthetic minimal medium containing 0.015 M uracil, and then washed twice again with deionized water.

Reaction conditions. Bleomycin was added to washed cells suspended at 10^7 cells per ml in deionized water. All reactions were carried out with aeration, and vessels were covered with foil. During the reaction incubation, cells were not exposed to nutrient conditions or growth medium because components in media can vary and affect reactions with bleomycin. Also, growth media contain Fe(II), and the quantities of Fe(II) can vary in growth media under different conditions. Yeast cells can be incubated in water for long periods without any detectable loss in DNA integrity or survival.

Bleomycin (Bristol Laboratories, Syracuse, N.Y.) was dissolved and diluted in the dark in deionized water (pH 5.6 to 6.7) just prior to use, and its A_{292} was monitored. At the end of each incubation, cells were diluted with cold deionized water, sedimented immediately, resuspended, incubated for 3 min in 0.01 M EDTA, and washed three times in cold 0.01 M EDTA. Control cells were handled in a manner identical to that of treated cells except that bleomycin was not added. Ferrous sulfate was added to defined synthetic medium (38). No precipitation was observed, indicating that ferrous iron was not oxidized in substantial amounts to the ferric form.

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Spheroplast formation. Washed cells were resuspended at approximately 10^8 cells per ml in 0.9 M sorbitol–0.1 M sodium citrate–0.05 M EDTA (pH 5.8; also see reference 8) containing 0.5 to 3 mg of Zymolyase 60000 per ml (Miles Laboratories, Evanston, Ill.). Following incubation at 34°C, spheroplasts were sedimented in a refrigerated RC5B SS34 rotor at very low speed and gently resuspended in cold 0.1 M sodium citrate–0.1 M EDTA. Approximately 5×10^6 to 1×10^7 spheroplasts in 0.1 ml were immediately layered onto the lysis layer on the density gradient.

Velocity sedimentation of DNAs and molecular weight determinations. Low-salt 5 and 20% (wt/vol) alkaline sucrose (ultrapure density gradient grade; Schwartz/Mann, Orangeburg, N.Y.) solutions contained 0.5 M NaCl, 0.01 M EDTA, and 0.2 M NaOH. Gradients were formed in 5-ml polyallomer ultracentrifuge tubes by the addition of 2.5 ml of 5% and 2.4 ml of 20% solutions to a custom-made gradient maker. Gradients were overlaid with 0.1 ml of a lysis buffer containing 1.3% purified sodium lauroylsarcosinate (97% minimum [Colgate-Palmolive Company]), 0.6 M NaCl, 0.2 M NaOH, and 0.067 M disodium EDTA. After gently layering the spheroplasts, 1 h was allowed for lysis of spheroplasts and unwinding of DNAs. For a sedimentation marker, T4 particles containing [2- 14 C]thymidine DNA were incubated in 2% Sarkosyl for 10 min at 60°C and layered on gradients. T7 [6- 3 H]thymidine DNA was used as a lower-molecular weight marker.

Nucleic acids were sedimented at 8,000 rpm for 19 to 19.5 h in an SW50.1 rotor (Beckman Instruments, Inc., Fullerton, Calif.) at 20°C. An average of 25 fractions of approximately 0.2 ml each was collected from the bottom of each gradient with the aid of a peristaltic pump (Buchler Instruments Division, Nuclear-Chicago Corp., Fort Lee, N.J.). Fractions were collected on Whatman no. 17 premarked paper strips (5). Paper strips were washed three times in 5% trichloroacetic acid and twice in 95% ethanol. Twelve to 24 h was allowed for each trichloroacetic acid wash to enable RNA hydrolyzed during alkaline sedimentation to diffuse from the paper strips. Filters were dried, and acid-insoluble radioactivity in each fraction was determined by liquid scintillation counting.

The gradient system was precalibrated with phage markers to establish the relationship of sedimentation distance, time, and force to molecular weight. This relationship was used to compute weight-average molecular weights from the amount of radioactivity sedimenting in different positions throughout the gradient. With T4 and T7 DNAs as standards, the gradient system was determined to be isokinetic, and the constant, k , and exponent, α , were experimentally determined for the Studier equation, $S = kM^\alpha$. With the aid of a computer program, weight-average molecular weights were calculated from distributions of radioactivity in gradients and related to number-average molecular weights (10, 21, 28, 35–37).

This assay detects DNA lesions which ultimately result in breaks, including alkali-labile sites, single-strand breaks, and double-strand breaks, under the described velocity sedimentation conditions in alkali. From the distributions of radiolabeled DNA under the established sedimentation conditions, the numbers of single-strand breaks (per 10^8 daltons) relative to the untreated control were calculated as follows:

$$10^8 \times \left[\frac{M_r(\text{untreated})/M_r(\text{treated}) - 1}{M_r(\text{untreated})/2} \right]$$

In this assay system, alkali-labile lesions left in DNA by the release of free bases by bleomycins are converted to DNA breaks and are included in the calculation of numbers of DNA breaks. Although certain assumptions were made and are

FIG. 1. Effect on sedimentation of cellular DNAs of coincubating cells with EDTA and bleomycin. Early-stationary-phase cells were resuspended after the chase in supplemented synthetic minimal medium containing 0.015 M uracil, held overnight at 4°C, washed three times with deionized water, and incubated for 20 min with 1.3×10^{-6} M bleomycin or 1.3×10^{-6} M bleomycin and 0.05 M EDTA in deionized water at 23°C. ■, DNAs from cells incubated for 20 min without bleomycin (control); △, DNAs from cells incubated with bleomycin; ○, DNAs from cells incubated with bleomycin and EDTA.

inherent in using this method for approximating the numbers of single-strand breaks, any error associated with the assumptions should be equivalent for DNAs from cells incubated with or without bleomycin.

RESULTS

EDTA inhibited DNA damage. We first sought to determine if metal ions performed a role in the production of cellular DNA lesions by bleomycin. This was investigated by comparing the molecular weights of DNAs carefully isolated from cells incubated for 20 min with bleomycin with the molecular weights of DNAs isolated from cells incubated for 20 min with bleomycin and EDTA. The size of native *S. cerevisiae* DNA is ideal for velocity sedimentation through sucrose, particularly at low centrifugal speeds. This assay is also quite sensitive and accurate, particularly under the limited reaction conditions required for the current study (27).

Typical distributions of radioactivity in sedimentation profiles of high-molecular weight DNAs from cells incubated without bleomycin (20 min) are compared in Fig. 1 with those of the low-molecular weight DNAs from cells incubated with bleomycin. The 80 to 90% of DNA from untreated *S. cerevisiae* which is nuclear in origin sediments at approximately 70 to 75% of the gradient in a major peak (ca. $M_r = 2 \times 10^8$ to 2.4×10^8 [Fig. 1]), while mitochondrial DNA from untreated cells sediments in the top 25% in a minor peak (Fig. 1) under the sedimentation conditions routinely used. When EDTA was added, DNA breaks were inhibited in proportion to increases in the concentrations of EDTA in the reaction mixture. In the presence of 0.05 M EDTA, DNA breaks were completely eliminated (Fig. 1).

Assay for residual DNA breakage or chemical activity producing DNA breaks during the lysis incubation. In order to detect breaks which could be introduced by residual bleomy-

TABLE 1. Analysis of molecular weights of DNAs on the gradient

Expt ^a	Molecular weight (cell type)			
	Single		Combined	
	¹⁴ C	³ H	¹⁴ C	³ H
A	19 × 10 ⁷ (untreated A364A)	9.2 × 10 ⁷ (treated H288)	18 × 10 ⁷ (untreated A364A)	9.9 × 10 ⁷ (treated H288)
B	9.2 × 10 ⁷ (treated A364A)	18 × 10 ⁷ (untreated A364A)	9.2 × 10 ⁷ (treated A364A)	18 × 10 ⁷ (untreated A364A)

^a Washed cells were suspended at 1 × 10⁷ to 2 × 10⁷ cells per ml in deionized water with or without bleomycin. After 20 min, EDTA was added to 5 × 10⁻² M. Cells were washed twice in 5 × 10⁻² M EDTA and then were converted to spheroplasts. Equal numbers of spheroplasts were layered on the lysis layer on the gradient.

cins or metabolic products of the glycopeptides on DNAs released from cells, molecular weights of DNAs were routinely measured during the lysis incubation just prior to DNA sedimentation. It was essential to establish that breaks were not introduced into DNAs at this step. To determine this, spheroplasts prepared from cells containing [2-¹⁴C]thymidine DNA were layered with spheroplasts prepared from cells containing [6-³H]thymidine DNA. One group of cells had been incubated with 6.7 × 10⁻⁷ M bleomycin, but the other group had not been incubated with bleomycin. If bleomycins or metabolic products from treated cells caused breakage of DNAs released from untreated cells, the DNAs from untreated cells would exhibit reduced molecular weights.

Table 1 summarizes results from two of these assays. Because the molecular weights of DNAs from untreated cells were the same whether the DNAs were sedimented alone or with DNAs from cells which had been incubated with bleomycin, we concluded that DNAs were not degraded during cell lysis or sedimentation.

EDTA-inhibited cell killing. That DNA damage was completely inhibited by EDTA establishes the role and importance of metal ions in the production of cellular DNA lesions. We also investigated the role of metal ions in cell killing by bleomycin under identical experimental conditions. As for DNA lesions, cell killing was inhibited in proportion to increases in the concentrations of EDTA added to each reaction mixture (6.7 × 10⁻⁷ M bleomycin and 1 × 10⁻⁶ to 5 × 10⁻² M EDTA [data not shown]). As illustrated in the representative experiment shown in Fig. 2, cell killing by 0.67 × 10⁻⁶ to 6.7 × 10⁻⁶ M bleomycin was completely eradicated in the presence of 0.05 M EDTA. Thus, the role and importance of metal ions are established not only in the production of intracellular lesions in DNA but also in cell killing.

Fe(II) supplementation during growth. We next considered the possibility that preloading cells with iron could affect the activities of the congeners. We reasoned that the cytotoxicity of bleomycin should increase if supplementary iron in the form of Fe(II) were provided to cells during their growth before the cells were exposed to bleomycin. This hypothesis was tested by growing cells for three generations (doublings) in defined synthetic growth medium to which ferrous sulfate was added and then incubating the cells with bleomycin.

The rationale for this experimental design is based upon two plasma membrane components involved in the uptake and transport of Fe(II) in *S. cerevisiae*. One system involves non-saturable uptake of ferrous iron (9). The existence of this uptake system and the fact that it is not saturable at concentrations of >3 × 10⁻⁵ M ferrous iron enabled us to use high Fe(II) concentrations in these experiments. In addition to this ferrous transport system, *S. cerevisiae* possesses a plasma ferric reductase system. The second system involves the reduction of insoluble ferric ions to soluble ferrous ions which are then transported to the cytoplasm of the cells. With both systems,

Fe(II) is stable in the reducing environment of the cytoplasm of the cell.

Figure 3 compares the cytotoxic activities of bleomycin on cells grown in medium to which ferrous sulfate was added with the cytotoxic activities of bleomycin on cells grown the same number of doublings in the same medium to which ferrous sulfate was not added. Cells were inoculated at 8 × 10⁶ cells per ml and grown for 41 h at 23°C in defined synthetic growth medium to which 0, 10⁻⁵, 10⁻⁴, or 10⁻³ M ferrous sulfate was added. Generation times were similar (3.3 to 3.5 generations) with or without the supplemental iron, indicating the addition of Fe(II) did not significantly alter doubling times or growth rates. This similarity in growth of each of the cell populations led to a similar dilution effect of the supplemental ferrous iron in each growing population. Cells were washed three times in deionized water prior to 20-min incubations with 1.3 × 10⁻⁶ M bleomycin in deionized water.

As Fig. 3 illustrates, the lethal effects of 1.3 × 10⁻⁶ M bleomycin on cells grown with supplemental Fe(II) were

FIG. 2. Effect on cell killing of coincubating cells with EDTA and bleomycin. Cells were grown and incubated under experimental conditions identical to those for DNA experiments (Fig. 1).

FIG. 3. Effect of supplementing cells with 10^{-5} M, 10^{-4} M, and 10^{-3} M ferrous iron on the subsequent killing of the cells by bleomycin. ▨, no bleomycin; ■, bleomycin. S/S_0 , surviving fraction supplemented with ferrous sulfate/surviving fraction not supplemented with ferrous sulfate. Error bars indicate standard errors calculated from means of 15 to 18 replicates.

greater than the lethal effects of the same concentration of bleomycin on cells grown without the supplemental Fe(II). Lethal effects of bleomycin were increased approximately 50% with 10^{-5} M Fe(II), almost twofold with 10^{-4} M Fe(II), and 2.8 times with 10^{-3} M Fe(II).

DISCUSSION

Fewer bleomycin molecules were required to kill cells grown in medium enriched with iron than were required to kill cells grown in the presence of normal amounts of iron. In approaching this study, we realized that cells may already contain adequate levels of iron. Moreover, a variety of metal chelating agents are endogenous to cells and are present during experimental growing conditions. In the current study, in fact, DNA damage and cell killing by bleomycin were completely inhibited by EDTA (Fig. 1 and 2) or strongly inhibited at low concentrations of EDTA (data not shown). Nevertheless, cell killing increased as a function of increasing the concentrations of Fe(II) supplied to cells during their growth (Fig. 3), indicating the cellular system provides the source of reducing power for the reactions or activities of bleomycin molecules.

This is the first report of bleomycin-treated cells which had been grown in the presence of increasing iron supplementation, and the responses of these cells predict a way of optimizing cytotoxic activities of bleomycin molecules. This experimental approach is quite different from coincubating ferrous iron, bleomycin, and cells (e.g., references 13 and 41). We have not determined if Fe(II) ions in the cells are bound to bleomycin or if Fe(II) affects cells in a way which facilitates but does not directly cause elevated numbers of DNA lesions or increased cell killing by the bleomycin congeners. Ferrous iron could affect a number of intracellular pathways directly related or unrelated to the chemical activation of bleomycin and degradation of DNA.

Ferric reductase activity (7, 20) and, possibly, another control of iron uptake (9) in *S. cerevisiae* are regulated by the concentrations of iron in growth media. The ferric reductase-ferrous transport system in *S. cerevisiae* (7, 9, 20) may provide a model of iron metabolism in mammalian cells (9).

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