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Inhibition of Bleomycin-induced DNA Breakage by Superoxide Dismutase[1]

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ABSTRACT

Inhibition of bleomycin (BLM)-induced DNA breakage by superoxide dismutase (SOD) has been reported and presumed to be due to its removal of the superoxide free radicals generated by BLM in the presence of iron(II). We have studied the possibility that the inhibitory effect might result from DNA-binding of SOD. The effect of copper-zinc SOD on BLM-induced DNA degradation was investigated using the PM-2 DNA fluorescence technique. PM-2 DNA was incubated with BLM in the presence or absence of native and heat-inactivated copper-zinc SOD as determined by the epinephrine autoxidation method. The concentrations of SOD required to inhibit 50% PM-2 DNA degradation for the native and the inactivated SOD were 100 and 120 μg/ml, respectively. Analysis of the reaction mixture by agarose gel electrophoresis confirmed the absence of DNA degradation by BLM in the presence of either form of SOD. PM-2 DNA was shown to bind native or inactivated SOD by Sephadex G-100 column chromatography, fluorescence-quenching studies, and agarose gel electrophoresis. Thus, these results indicate that SOD is able to bind to PM-2 DNA and inhibit BLM-induced degradation independently of its free radical-scavenging activity. The inhibition was more effective against BLM than other compounds which degrade PM-2 DNA. This suggests that SOD may bind to BLM-binding and/or BLM degradation sites in PM-2 DNA, and the observed inhibition is unrelated to its effects on free radicals.

INTRODUCTION

BLM[4] is a glycopeptide antitumor antibiotic isolated from *Streptomyces verticillus* (19). Blenoxane, the BLM preparation used clinically, has proven effective in the treatment of cancers of different types including squamous-cell carcinomas, testicular carcinomas, and lymphomas (1, 3). The most serious dose-limiting side effect has been the development of pulmonary toxicities observed at relatively high doses in older patients (3). This has led to the synthesis of several new structural analogs having improved therapeutic indices (15). Studies of the mechanism of action of BLM have provided evidence suggesting that the cytotoxicity is primarily due to the degradative effects which the drug has on DNA (3, 5). Several investigators have reported results indicating that the DNA strand scission by BLM occurs at specific sites on the DNA (4, 9–11, 13, 18). More recently, workers concluded from studies in which the reaction products of BLM and DNA in the presence of iron(II) and O_2 were analyzed that the strand cleavage results from the oxidation of the C-3′—C-4′ bond of the deoxyribose (2, 17). It has been suggested that the breakage of DNA by BLM might be mediated by the reactivities of oxygen-derived free radicals and that the inhibition of BLM DNA breakage by SOD results from scavenging of these free radicals (11, 16).

Results of the present study suggest that the inhibition of BLM-induced DNA degradation by SOD may be due to binding of the protein to DNA which prevents BLM from binding to and degrading DNA.

MATERIALS AND METHODS

Chemicals. The chemicals were reagent grade quality from Fisher Scientific Co. (Pittsburgh, Pa.). Bovine copper-zinc SOD, DNase I, epinephrine, and ethidium bromide were obtained from Sigma Chemical Co. (St. Louis, Mo.). Auromomycin, Blenoxane (BLM), and neocarzinostatin were provided by Bristol Laboratories (Syracuse, N. Y.). *Pseudomonas* bacteriophage (PM-2) DNA was purified by the method described previously (7).

Assay Procedures. The PM-2 DNA fluorescence assay used to measure DNA breakage activity has been described in a previous report (6, 7). The assay was performed by adding drug or enzyme preparation to 50 μg PM-2 DNA in buffer (0.5 M sodium borate, pH 9.5:20 mM 2-mercaptoethanol) to give a final volume of 500 μl. Incubations were carried out for 30 min at 37°. Triplicate aliquots (0.1 ml) of the assay solution were then placed in 0.9 ml denaturation buffer (pH 12.1) followed by the addition of 0.1 ml ethidium bromide (22 μg/ml in denaturation buffer). Using an Aminco-Bowman spectrophotofluorometer, the fluorescence was measured at excitation and emission wavelengths of 530 and 590 nm, respectively.

The epinephrine autoxidation assay for measuring enzymatic activity of SOD was performed as described by Misra and Fridovich (14). This method is based on the ability of SOD to inhibit the autoxidation of epinephrine at alkaline pH. Since the oxidation of epinephrine leads to the production a of pink adrenochrome, the rate of increase of the absorbance at 480 nm, which represents the rate of autoxidation of epinephrine, can be conveniently followed. SOD has been found to inhibit this radical-mediated process. Epinephrine (50 μl of 0.55% epinephrine in HCl, pH 2.0) was added to SOD preparations in buffer (0.05 M Na_2CO_3, pH 10.2) to make a final volume of 2.2 ml. The absorbance at 480 nm was measured at 15-sec intervals for 15 min at room temperature.

Chromatography. The Sephadex columns (Pharmacia Fine Chemicals, Piscataway, N.J.) (88 x 1.5 cm) were run at a flow rate of 6 ml/hr using a Na_2HPO_4:KH_2PO_4 buffer (0.007 M, pH 6.8). Eluates (3 ml/tube) were collected with a Gilson fraction collector (Gilson Medical Electronics, Inc., Middleton, Wis.), and the absorbance at 230 and 260 nm was measured. The absorbance peaks were pooled and dialyzed against 2 liters H_2O for 24 hr while sitrring at 4°. The fractions were lyophilized after freezing the samples in an acetone:dry ice bath. The dried samples were resuspended in deionized water, and the protein concentration was determined by the method of Lowry *et al.* (12).

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[3] Recipient of NIH Grant CA-10893.
[4] The abbreviations used are: BLM, bleomycin; SOD, superoxide dismutase; IC_{50}, concentration of superoxide dismutase required to inhibit 50% DNA degradation; EC_{50}, effective drug concentration causing 50% breakage of DNA.
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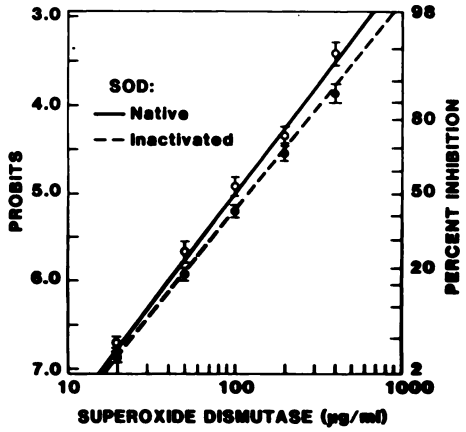

Chart 1. Effect of native and heat-inactivated SOD on BLM-induced degradation of PM-2 DNA. Native and heat-inactivated SOD were tested using a standardized PM-2 DNA fluorescence assay for BLM. The reaction mixture contained 100 ng Blenoxane per ml and native or inactivated SOD (20, 50, 100, 200 or 400 μg/ml) in a sodium borate buffer (0.5 M, pH 8.5) that contained 20 mM 2-mercaptoethanol. The reaction was initiated by the addition of 25 μg Form I PM-2 DNA per ml and incubated at 37° for 30 min. The reaction was stopped by the addition of 0.9 ml of denaturation buffer (pH 12.1) to 100 μl of the reaction mixture. Ethidium bromide (final concentration, 2.2 μg/ml) was added, and the fluorescence was measured at 530-nm excitation and 590-nm emission. *Abscissa*, SOD concentration; *ordinate*, percentage of inhibition of BLM-induced DNA breakage.

Aliquots of the different fractions were assayed for SOD activity by the epinephrine autoxidation assay and for inhibition of BLM-induced DNA degradation by the PM-2 DNA fluorescence assay.

Gel Electrophoresis. The degradation of PM-2 DNA induced by BLM and the effect of native and heat-inactivated SOD were analyzed by agarose gel electrophoresis as described previously (7). Agarose gels (0.9%) in Tris-HCl buffer (0.05 M, pH 7.8) were prepared in horizontal plastic glass containers (20 x 20 cm). Aliquots of the reaction mixtures containing 1.5 μg DNA were run at 150 V, 20 ma, for 9 hr. The gels were then stained with ethidium bromide (5 μg/ml), inspected under a UV mineral lamp, and photographed using a Polaroid CU-5 Land Camera.

RESULTS

Chart 1 shows the effect of native and heat-inactivated bovine copper-zinc SOD on BLM-induced PM-2 DNA degradation. Various protein concentrations (20, 50, 100, 200, and 400 μg/ml) of SOD were incubated with BLM (70 nM) and PM-2 DNA (50 μg/ml) in a sodium borate buffer (0.05 M, pH 9.5 to 5) containing 20 mM 2-mercaptoethanol at 37° for 30 min. The DNA degradation induced by BLM, indicated by the decrease of ethidium bromide fluorescence, was inhibited to a similar extent by increasing concentrations of both the native and the heat-inactivated SOD. The IC_{50} values for the native and the heat-inactivated SOD were 100 and 120 μg/ml, respectively.

Analysis of the reaction mixtures by agarose gel electrophoresis, shown in Fig. 1, indicated that the BLM-induced degradation of Form I PM-2 DNA (covalently closed circular DNA) seen in *Lane B* was inhibited by the presence of native SOD or heat-inactivated SOD (*Lanes C* and *D*). *Lanes E* and *F* show that neither preparation of SOD had any degradative effect on the DNA. The Form I DNA appears essentially the same as in control (*Lane A*).

Heat inactivation of copper-zinc SOD required boiling for 10 min by holding the sample tube directly over an open flame. Chart 2 gives the results obtained from epinephrine autoxidation assays of native and heated copper-zinc SOD. The enzyme preparations were exhaustively dialyzed prior to the determination of the enzymatic activities. The results of the assays showed clearly that the heated copper-zinc SOD did not have any effect on the production of adrenochrome. The absorbance maximum at 480 nm and the reaction rate were essentially the same as those obtained in the control reaction which did not contain any SOD. The native copper-zinc SOD had a marked inhibitory effect on the autoxidation reaction. Thus, heat-inactivated SOD was not capable of scavenging the superoxide free radical.

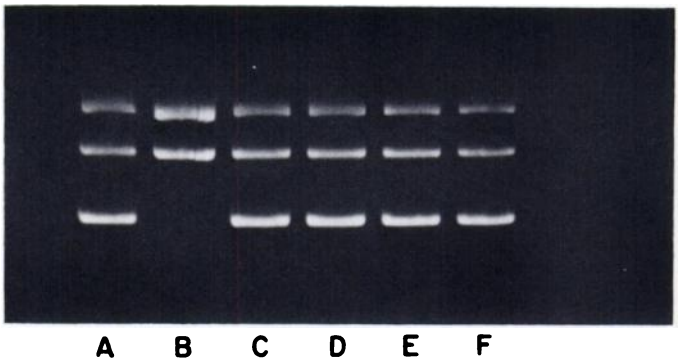

Fig. 1. Samples of reaction mixtures from PM-2 DNA fluorescence assay showing the inhibition at BLM-induced DNA breakage by native and inactivated SOD. *Lane A*, DNA control (1.5 μg); *Lane B*, BLM (70 nM); *Lane C*, BLM + native SOD (200 μg/ml); *Lane D*, BLM + inactivated SOD (200 μg/ml); *Lane E*, native SOD (200 μg/ml); *Lane F*, inactivated SOD (200 μg/ml).

The physical binding of SOD to DNA was demonstrated by gel filtration column chromatography. Either native (250 μg) or heat-inactivated (250 μg) SOD was mixed with an equal amount of PM-2 DNA (250 μg). The mixtures were then incubated 1 hr at room temperature and applied to a column of Sephadex G-100 (88 cm x 1.5 cm). Chart 3 shows the elution patterns of the free DNA and SOD and the mixtures. The PM-2 DNA was eluted in the void volume (Chart 3 *A*). The native and heated SOD were retained in the gel and eluted in the same fraction (Chart 3, *B* and *D*). The native and the heated SOD when mixed with the PM-2 DNA were eluted in the void volume with the DNA, indicating the formation of a stable complex of SOD and DNA (Chart 3, *C* and *E*).

Further evidence of DNA binding was provided by the results of fluorescence-quenching experiments. Chart 4 shows that the fluorescence of native SOD or heat-inactivated SOD was quenched by increasing concentrations of calf thymus DNA. The fluorescence-quenching effect was greater against the native SOD than the inactivated SOD and was less for both preparations in the presence of 2-mercaptoethanol.

To exclude the possibility that SOD inhibited BLM-induced DNA breakage by binding to and inactivating BLM, tritium-labeled BLM (bleomycin A_2) was mixed with serum and applied to a Sephadex G-25 column. No radioactivity was detected in the void fraction representing a molecular weight fraction of serum proteins which includes SOD. All of the radioactivity was eluted in a subsequent fraction which contained the BLM (data not shown).

To determine whether the inhibitory effect of SOD is specific to BLM, the effect of SOD on the activity of other DNA-degrading agents such as neocarzinostatin, auromomycin, and DNase I were studied. The DNA degradation induced by each of these

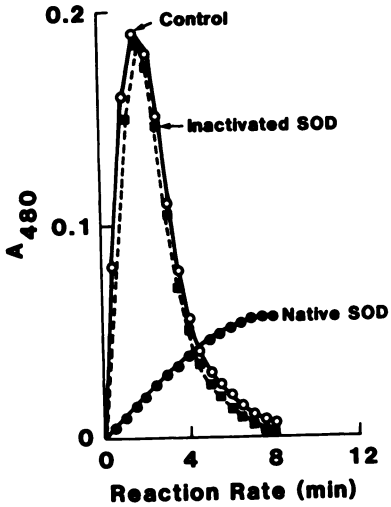

Chart 2. Epinephrine autoxidation assay for SOD free radical-scavenging activity. The autoxidation of epinephrine to adrenochrome was determined by measuring the absorbance at 480 nm. The reaction was performed in 2.2 ml Na_2CO_3 (0.05 M, pH 10.2) at room temperature. ○, epinephrine control (0.69 mM); ●, native copper-zinc SOD (227 μg/ml); ■, heat-inactivated copper-zinc SOD (227 μg/ml).

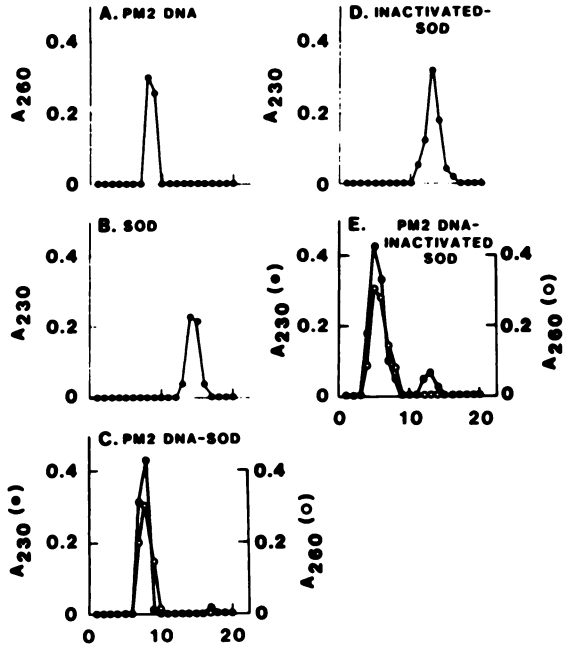

Chart 3. The binding of SOD to PM-2 DNA was determined by gel filtration column chromatography. PM-2 DNA, SOD, and heat-inactivated SOD were applied on a Sephadex G-100 column (88 x 1.5 cm) and eluted with $NaKPO_4$ buffer (0.007 M, pH 6.8). Elution patterns were monitored at 260 and 230 nm. *A*, PM-2 DNA (250 μg); *B*, native copper-zinc SOD (100 μg); *C*, PM-2 DNA (250 μg) complexed to native copper-zinc SOD (250 μg); *D*, heat-inactivated copper-zinc SOD (250 μg); *E*, PM-2 DNA (250 μg) complexed to heat-inactivated copper-zinc SOD (250 μg).

agents was determined by use of the PM-2 DNA fluorescence technique. The results of the assays were expressed by probit-log plots (8) of the percentage of fluorescence decrease *versus* the drug concentrations. From the probit graphs, EC_{50} values were obtained. The EC_{50} is the effective drug concentration causing 50% breakage of the DNA under the standard conditions of the assay for each agent. The EC_{50} value of each agent was then used to determine the IC_{50} value for SOD under the

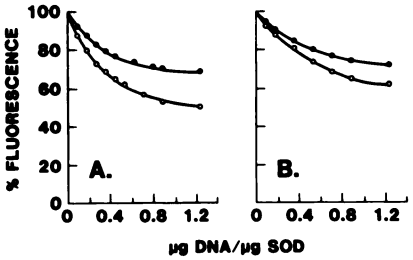

Chart 4. Fluorescence-quenching effect of DNA on SOD in the absence (*A*) or presence of 20 μM 2-mercaptoethanol. Fluorescence at 360 nm was measured with an excitation at 200 nm in a 1-ml 50 mM borate buffer (pH 9.5) containing 250 μg native (○) or heat-inactivated (●) SOD. Concentrated calf thymus DNA (1.1 mg/ml) was used for titration.

standardized conditions of the assay. The IC_{50} value for SOD is the concentration of SOD required to inhibit 50% of the DNA breakage induced by the respective agent using the EC_{50} as the standard drug concentration. The results of the experiment are shown in Table 1. The IC_{50} value for BLM was less than that of neocarzinostatin, DNase I, or auromomycin. These results suggested the inhibitory effect by SOD was not specific to but relatively greater for the DNA cleavage induced by BLM.

To exclude the possibility that SOD might have inhibited the degradation of DNA by BLM by binding Fe(II) in the reaction mixture, the removal of which would cause a decrease of the DNA degradation activity of BLM, the effects on BLM-induced DNA degradation of various concentrations of $FeCl_2$ were tested in the presence and absence of native and inactivated SOD. The results are shown in Table 2. The inhibitory activity of the SOD was not appreciably affected even at Fe(II) molar concentrations greater than 30-fold in excess of the protein. This indicated that the inhibition by SOD was not due to removal of adventitious amounts of Fe(II) which have been shown to be present in preparations of Blenoxane and DNA and to participate in DNA degradation induced by BLM (8).

Table 1

SOD inhibition of DNA degradation induced by several agents

Agent	EC_{50}	IC_{50} (μM)
BLM	0.5 nM	2.1
Neocarzinostatin	10 μM	3.9
DNase I	2.3 nM	5.4
Auromomycin	16 μM	7.8

Table 2

Effect of ferrous chloride on SOD inhibition of PM-2 DNA degradation induced by BLM

Blenoxane (26.0 nM) and ferrous chloride (0.1 to 100 μM) were incubated 30 min at 37° in the presence and absence of native and heat-inactivated SOD (Orgotein) (3 μM) with PM-2 DNA (1.0 A_{260} unit) in sodium borate buffer (0.05 M, pH 9.5) containing 25 nM 2-mercaptoethanol. Aliquots (100 μl) of the reaction mixture were added to denaturation buffer (900 μl, pH 12.1) and ethidium bromide (100 μl; 22 μg/ml). The fluorescence was measured at 530 nm excitation and 590 nm emission wavelengths. The percentage of inhibition was calculated relative to values of control reactions which did not contain SOD.

	BLM-induced DNA breakage (% of inhibition)	
$FeCl_2$ (μM)	Native SOD	Inactivated SOD
0	72.6 ± 3.6[a]	90.4 ± 3.5
0.1	78.6 ± 4.8	84.3 ± 1.4
1.0	78.2 ± 2.5	98.7 ± 1.2
10.0	85.2 ± 2.9	100.0 ± 1.0
100	62.6 ± 1.8	77.4 ± 3.0

[a] Mean ± S.E.

DISCUSSION

SOD causes an inhibition of the degradation of DNA that is induced by BLM. This inhibitory effect has been presumed to be due to the scavenging of superoxide free radicals by the enzyme (11, 16). However, the results of the experiments described herein suggest the observed inhibitory effect was not due to the scavenging of superoxide but more likely to the physical binding of the enzyme to DNA which interfered with the interaction of BLM with DNA. The conclusion that SOD inhibits the degradation of DNA by a steric hindrance effect is not incompatible with reported information regarding the underlying mechanism of DNA strand scission induced by BLM. Sufficient evidence has accumulated to support an updated hypothesis (5) in which it is proposed that the DNA strand scission by BLM involves a 2-stage mechanism. In the first stage, BLM binds to DNA by ionic attraction mediated through the terminal amine and partial intercalation by its bithiazole moiety. This is followed by the second stage which involves the generation of reactive oxygen-derived free radicals by the ferrous binding site of BLM and the resulting oxidative cleavage of the DNA chain. Since the oxygen-derived free radicals detected in the BLM-DNA reaction have included the superoxide anion, a logical deduction was that the enzyme inhibited the reaction by its effect on the second stage, that is, by scavenging the superoxide free radical. The alternative possibility that the enzyme could inhibit the reaction by interfering with the binding of BLM to the DNA heretofore had not been reported. The results of our experiments (Chart 1) showed that the inactivated SOD was equally effective as the native enzyme with respect to the inhibition of the BLM-induced degradation of DNA and that the inhibitory effect is probably not due to the scavenging of the superoxide free radical. The fact that the enzyme was bound to DNA (Chart 3) would suggest an effect at the first stage by inhibiting the binding of BLM to the DNA.

In addition to the modes of inhibition already mentioned, other possible ones were considered. Since SOD is a metalloenzyme that binds metal ions known to bind BLM and some of which can either inhibit (Cu^{2+}) or potentiate (Fe^{2+}) its DNA-degradative activity, the enzyme preparations were dialyzed exhaustively to remove any free metal ions that might have become dissociated from the protein. The possibility that SOD was perhaps acting by chelation of ferrous ions present in trace amounts in the reaction mixture is not a likely one since the inhibition was not affected by the addition of known quantities of ferrous chloride. That the inhibition might have resulted from the binding of SOD directly to BLM was considered to be rather remote in view of other studies in which it was found that tritium-labeled bleomycin A_2 was not bound to serum proteins containing SOD.

It has been reported (4, 9, 13) that BLM induces DNA strand scission at specific neucleotide sequences. In particular, BLM has been shown to cause the release of pyrimidines (thymine or cytosine) when these bases are located next to a guanine at its 3′ side. The presence of BLM-specific sites on DNA has been confirmed by other workers (10) who were able to map the cleavage sites on PM-2 DNA. In light of these findings, the results of our experiments (Table 1) which indicated a relatively greater inhibitory effect for BLM suggest that SOD may bind to nucleotide sequences preferred by BLM.

The lack of effect by $FeCl_2$ on the inhibition of the BLM-induced degradation of PM-2 DNA to SOD shown by ethidium fluorescence technique was confirmed by agarose gel electrophoresis. Moreover, we observed that the nonspecific DNA degradation induced by high molar concentrations of Fe(II) was not inhibited by either the native or inactivated SOD (data not shown). This offers further confirmation that the inhibition of DNA degradation by SOD is unlikely to be related to Fe(II) binding by SOD.

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