Enhancement of DNA Chain Breakage by Bleomycin A₂ in the Presence of Microsomes and Reduced Nicotinamide Adenine Dinucleotide Phosphate¹

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ABSTRACT

Chain breakage in DNA induced by bleomycin A₂ (BLM) was enhanced more than 150-fold by the reduced nicotinamide adenine dinucleotide phosphate-dependent electron transport system of rat liver microsomes. However, the enhancement effect on DNA was partially reduced by the preincubation of BLM with the microsomal systems. BLM-Cu²⁺ was found to stimulate considerably microsomal reduced nicotinamide adenine dinucleotide phosphate-dependent oxygen consumption and malondialdehyde formation, whereas BLM inhibited both of the effects. These findings suggest that the pharmacological action of BLM is strongly affected by a membrane system, such as microsomes, that produces free radicals.

INTRODUCTION

Therapeutic effects of BLM, a water-soluble basic glycopeptide antitumor antibiotic (13, 36, 37), have been widely accepted in treatment against human squamous cell carcinoma and malignant lymphoma (10, 11, 14, 38). Extensive studies indicate that the mechanism of antitumor action of BLM is chain breakage in DNA (6, 19, 32). It has been amply documented that the DNA chain breakage by BLM is stimulated by free radical-producing systems, such as sulfhydryl compounds (19, 20), Superoxide anión (12), chain-breaking systems, that produces free radicals.

MATERIALS AND METHODS

Chemicals. BLM and BLM-Cu²⁺ (Lot Cm-26; hydrochloride salt; BLM:Cu²⁺ = 1) were supplied by Nihon Kayaku Co. Ltd., Tokyo, Japan. Tinoridine [2-amino-3-ethyloxycarbonyl-6-benzyl-4,5,6,7-tetrahydrothieno(2,3-c)pyridine hydrochloride] was obtained from Yoshitomi Pharmaceutical Indst. Ltd., Osaka, Japan. NADPH was obtained from Sigma Chemical Co., St. Louis, Mo. Calf thymus DNA was obtained from Worthington Biochemical Corp., Freehold, N. J.

RESULTS

Stimulation by BLM of DNA Chain Breakage by Microsomal NADPH-dependent Electron Transport System. The results in Table 1 clearly show that DNA was only slightly fragmented by BLM or by the NADPH-dependent microsomal electron transport system but that BLM, when combined with NADPH and microsomes, resulted in considerable DNA chain breakage. In fact this BLM-NADPH-microsomes system enhanced DNA chain breakage more than...
Cu2* was restored to some extent by 12 mM GSH.  

The somal electron transport system did not promote breakage of DNA chain breakage. Somewhat effective in decreasing the enhancement of DNA was explored. Superoxide dismutase was an enzyme known to scavenge the radical (18), was explored. Superoxide dismutase was served in the presence of 12 mM GSH or 40 μM tinoridine.

Since Superoxide anion is considered to be a participant in the microsomal electron transport (7), almost completely the DNA chain breakage was dependent on the concentration of BLM and/or microsomes (data not shown), and the percentage of DNA chain breakage was calculated as acid-solubilized radioactivity against initially added radioactive DNA.

150-fold compared to the system of BLM and microsomes. Yet omission of either NADPH or microsomes abolished this stimulation. The enhancing effect of microsomes was not replaced by heat-denatured microsomes. The rate of the DNA chain breakage was dependent on the concentration of BLM and/or microsomes (data not shown), and the addition of 0.2 mM p-chloromercuribenzoic acid, an inhibitor of microsomal electron transport (7), almost completely abolished the stimulation of DNA chain breakage. The effect of antioxidants on DNA chain breakage stimulated by the microsomal system was examined. The possible effects of GSH, known to inhibit lipid peroxidation reactions at high concentration (9), and tinoridine, an anti-inflammatory drug having antioxidant properties (23, 30), were also studied. No marked alteration in the stimulation of chain breakage in the BLM-NADPH-microsome system was observed in the presence of 12 mM GSH or 40 μM tinoridine. Since superoxide anion is considered to be a participant in microsomal free radical reactions (26), the potential effect of superoxide dismutase, an enzyme known to scavenge this radical (18), was explored. Superoxide dismutase was somewhat effective in decreasing the enhancement of DNA chain breakage.

In the case of BLM-Cu2+, the NADPH-dependent microsomal electron transport system did not promote breakage as effectively as in the case of BLM. The breakage by BLM-Cu2+ was restored to some extent by 12 mM GSH.

### Table 1

<table>
<thead>
<tr>
<th>Additions</th>
<th>cpm</th>
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</thead>
<tbody>
<tr>
<td>- NADPH</td>
<td>4.0</td>
</tr>
<tr>
<td>- NADPH + 50 μg BLM/ml</td>
<td>48.45</td>
</tr>
<tr>
<td>None</td>
<td>19.0</td>
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<tr>
<td>12 mM GSH</td>
<td>75.56</td>
</tr>
<tr>
<td>40 μM tinoridine</td>
<td>86.2</td>
</tr>
<tr>
<td>50 μg BLM/ml - microsomes</td>
<td>10.8</td>
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<tr>
<td>50 μg BLM/ml</td>
<td>77.56</td>
</tr>
<tr>
<td>50 μg BLM/ml + 12 mM GSH</td>
<td>74.8</td>
</tr>
<tr>
<td>50 μg BLM/ml + 40 μM tinoridine</td>
<td>89.03</td>
</tr>
<tr>
<td>50 μg BLM/ml + 0.2 mM p-</td>
<td>41.0</td>
</tr>
<tr>
<td>chloromercuribenzoic acid</td>
<td></td>
</tr>
<tr>
<td>50 μg BLM/ml + 10 μg/ml superoxide dismutase</td>
<td>6880.0</td>
</tr>
</tbody>
</table>

* Corresponding heat-denatured microsomes (98°; 15 min) were used instead of microsomes.

### Table 2

<table>
<thead>
<tr>
<th>No.</th>
<th>Addition at preincubation</th>
<th>Additions for DNA chain breakage</th>
<th>% of DNA chain breakage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>- NADPH</td>
<td>0.48 mM NADPH + 50 μg BLM/ml</td>
<td>78.4</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>0.24 mM NADPH + 50 μg BLM/ml</td>
<td>73.1</td>
</tr>
<tr>
<td>3</td>
<td>50 μg BLM/ml</td>
<td>0.24 mM NADPH</td>
<td>64.0</td>
</tr>
<tr>
<td>4</td>
<td>50 μg BLM-Cu2+/ml</td>
<td>0.24 mM NADPH</td>
<td>62.2</td>
</tr>
<tr>
<td>5</td>
<td>50 μg BLM-Cu2+/ml + 10 μM tinoridine</td>
<td>0.24 mM NADPH + 50 μg BLM/ml</td>
<td>64.0</td>
</tr>
</tbody>
</table>

### Table 1

**Stimulation of DNA chain breakage by microsomal NADPH-dependent electron transport system with BLM**

- The chain breakage in [3H]thymine-labeled DNA was estimated by acid-soluble radioactivity as described in "Materials and Methods." The standard reaction system contained microsomes (0.2 mg protein per ml), 0.24 mM NADPH, 16 μg [3H]thymine-labeled DNA per ml, 0.12 M KCl, and 40 mM potassium phosphate buffer (pH 7.4) at 37° (total volume, 0.5 ml). Additional components are shown in the table. After 30 min incubation the reaction was stopped by the addition of 0.1 ml 35% trichloroacetic acid. cpm denotes the acid-soluble radioactivity. The percentage of DNA chain breakage was estimated as acid-solubilized radioactivity against initially added radioactive DNA.

### Effect of preincubation of BLM with microsomes on DNA chain breakage

- Each reaction system at preincubation contained microsomes (0.2 mg protein per ml), 0.24 mM NADPH, 16 μg [3H]thymine-labeled DNA per ml, 0.12 M KCl, and 40 mM potassium phosphate buffer (pH 7.4) at 37°, with the indicated additions in the table (total volume, 0.5 ml). After 30 min preincubation the following reagents were added with 16 μg [3H]thymine-labeled DNA to induce chain breakage in DNA for 30 min. Acid-soluble radioactivity was estimated as described in Table 1. The percentage of DNA chain breakage was calculated as acid-solubilized radioactivity against initially added radioactive DNA.
rate of the oxygen consumption, whereas the same amount of BLM-Cu²⁺ stimulated the reaction rate. In place of BLM-Cu²⁺, the Cu²⁺ ion at comparable concentrations had no effect, as reported by Hochstein et al. (8).

The formation of malondialdehyde, an indication of microsomal NADPH-dependent lipid peroxidation reaction, was expressed as the quantity of TBA reactant (2). Addition of BLM (50 μg/ml) reduced the amount of TBA reactant formed, whereas BLM-Cu²⁺ resulted in an increase (Chart 2). Tinoridine diminished the BLM-Cu²⁺ effect.

The previous facts indicate that the microsomal lipid peroxidation reaction, which damages membranes (2), was stimulated by BLM-Cu²⁺ and that tinoridine inhibited the reaction effectively.

DISCUSSION

The results of this investigation have shown that the breakage of DNA by BLM was greatly enhanced by the NADPH-dependent electron transport system of microsomes. Low concentrations of iron salts in the presence of a reducing agent, such as 2-mercaptoethanol, stimulate the breakage of DNA by BLM (29). However, Fe³⁺, which is free in solution, is not an electron acceptor from NADPH (24), and heat-denatured microsomes were not effective to enhance the DNA chain breakage by BLM (Table 1). This evidence indicates that microsomes are not simply a source of ferric and/or ferrous ions. BLM has been known to form a chelate with Cu²⁺ in vivo as well as in vitro (33, 34). The inactivity of BLM-Cu²⁺ in our experiment agreed with other reports that the action of BLM for the DNA breakage is inhibited by chelating divalent cations, such as Cu²⁺ and Co²⁺ (22, 25, 31).

Current discussion has indicated that free radicals are involved in the reaction of the NADPH-dependent electron transport system of microsomes (7, 8, 24, 27, 28). Therefore it is quite reasonable to consider that certain types of radicals are involved in the enhancement of the breakage. Yet identifying the type of radicals was a problem because radical scavengers, such as tinoridine, GSH, or superoxide dismutase, were not effective. For the concentrations used in the DNA chain breakage experiment (see Table 1), tinoridine was sufficient to inhibit both the malondialdehyde formation accompanied by lipid peroxidation reaction (Chart 2) and the oxygen consumption (data not shown).

Ultrastructural studies revealed that marked alterations in the nucleus and cytoplasm were induced by BLM (1, 15). Microsomal membranes surround the nucleus, and radioautographic studies indicated that BLM was located mainly on the nuclear membrane and in the cytoplasm (4). The previously mentioned facts have suggested that nuclear DNA chain breakage induced by BLM may be enhanced by the microsomal electron transport system in living cells.

BLM was inactivated by radical-producing compounds, such as sulfhydryl compounds and peroxide, which enhanced the reaction of BLM with DNA (35). In agreement with this fact, BLM had the tendency to be inactivated by the microsomal system in this study.

BLM inhibited the microsomal NADPH-dependent lipid peroxidation reaction, such as tinoridine (Charts 1 and 2). In contrast, BLM-Cu²⁺ was found to stimulate the peroxidation reaction, and this reaction was effectively inhibited by tinoridine. As tinoridine was without effect to the enhancement of DNA breakage by BLM and the NADPH-dependent microsomal electron transport system, BLM was indicated to interact with the microsomal free radical-producing system before the sites of tinoridine and/or BLM-Cu²⁺ and might be activated to stimulate DNA chain breakage. More details about BLM-Cu²⁺ and the relationship of BLM to malondialdehyde formation are now under investigation.

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REFERENCES

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