Isolation and Characterization of Bleomycin-sensitive Chinese Hamster Ovary Cells

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ABSTRACT

Nineteen bleomycin-sensitive Chinese hamster ovary cell mutants have been isolated using a replica plating and photography approach. As judged by the dose which reduces cell survival to 37% of the untreated control, these mutants are from 2.5- to 32-fold more sensitive to a 16-h bleomycin treatment than the parental cell, while for chronic bleomycin exposure, the increase in sensitivity was 5 to 58 times that of the parental cell. Four bleomycin-sensitive mutants had increased sensitivities to killing by γ-rays (2- to 3-fold), mitomycin C (2-fold), and ethyl methane sulfonate (4- to 5-fold), while six other mutants were resistant to these agents. Nine other bleomycin-sensitive mutants displayed a variable pattern of cross-sensitivities to these agents. Using the technique of alkaline elution, the relative frequency of single-strand DNA breaks introduced by varying concentrations of bleomycin was examined in one mutant and its parent cell. The elution profiles of both cells were similar, suggesting that the bleomycin sensitivity of this mutant is not due to a greater frequency of single-strand breaks introduced by bleomycin.

INTRODUCTION

In yeast and bacteria, the availability of mutants which are hypersensitive to killing by specific DNA-damaging agents and deficient in particular repair pathways has been a vital factor in elucidating the molecular mechanisms of DNA repair and their role in mutagenesis and cellular lethality. In human cells, much of what is known about DNA repair has been obtained from the study of cells isolated from patients with rare genetic diseases such as XP, AT, and Fanconi’s anemia. Cells from these patients are abnormally sensitive to killing by specific mutagens, and at least one of these XP-derived mutant cell lines is defective in a specific DNA repair pathway (1). Further, patients with these diseases have a much higher risk of developing cancer, which strongly suggests a linkage between DNA repair deficiency and human carcinogenesis (5). The genetic complexity of these diseases is indicated by the finding that there are eight complementation groups in XP (6) and five groups in AT cells (7, 8). However, despite this relatively large number of complementation groups, the types of mutants that can be obtained from this source are limited to those that allow near-normal development of the fetus and can be detected clinically. Thus, many types of interesting repair-defective cells probably cannot be obtained from this source. As an alternative to this approach, we and others have developed techniques for isolating mouse and hamster cell lines that are hypersensitive to killing by various physical and chemical agents (9-19). In this report, we describe the isolation of 19 mutants in the Chinese hamster cell line which are extremely sensitive to killing by the antitumor drug bleomycin. These mutants have differing cross-sensitivities to other DNA-damaging agents such as γ-rays, mitomycin C, ethyl methane sulfonate, and ICR-191 and may represent new classes of repair-deficient mutants. A study of the biochemical basis for the increased sensitivity of these mutants could well provide important insights into the mechanism for the cytotoxic activity of bleomycin toward tumor cells. This information could be extremely important for the development of new and more effective antitumor agents.

MATERIALS AND METHODS

Cell Culture. The CHO cell line, CHO-K1, used for these studies was routinely cultured in Ham’s F-12 medium supplemented with 10% fetal calf serum, 2 μM glutamine, 200 units/ml penicillin, and 100 μg/ml streptomycin. Incubations were at 37.5°C in a 5% CO2 atmosphere.

Mutant Isolation. Bleomycin-sensitive mutants were isolated using a modification of the dark-field photography method described earlier (20). Cell populations were mutagenized by a 16-h treatment with either EMS (300 μg/ml) or ICR-191 (4 μg/ml) and subcultured for 5 days to allow for recovery from the mutagen treatment. Aliquots of these mutagen-treated cells were plated into a series of 100-mm tissue culture plates to give 200 to 300 colonies per plate. After 5 to 6 days of culture, a nylon cloth replica of the colony pattern on each plate was produced, and the plates were rinsed with PBS to remove loose cells and incubated overnight in growth medium to allow for recovery from the replica procedure (19). The plates were again rinsed, given medium containing either 0.4 to 1.2 μg/ml bleomycin for an overnight exposure (16 h) or 0.125 to 0.5 μg/ml bleomycin for chronic treatment, and photographed using dark-field illumination as described previously (19, 20). The following day, plates receiving an overnight bleomycin treatment were rinsed, and fresh growth medium was added. After 3 days (5 days for chronic treatment) of colony growth, the plates were again photographed, and bleomycin-sensitive colonies were identified by superimposing the negative from the first photograph over the print from the second photograph. Colonies that continued to grow after bleomycin treatment have a white halo surrounding a black center dot, while prospective bleomycin-sensitive colonies had either no halo or only a small one. Cells from these colonies were isolated from daughter colonies on the nylon cloth replica (19, 20), and subclones were tested for bleomycin sensitivity. In some cases viable cells could be obtained directly from the bleomycin-treated colony. With this bleomycin treatment schedule, in which cells were treated for one or more generations, a cell would receive bleomycin-induced damage in all phases of the cell cycle. Thus a colony containing mutant cells that are abnormally sensitive in only one phase of the cell cycle would still receive a lethal dose and therefore could be identified. Further, these exposure times allow the intracellular concentration of bleomycin to reach steady-state levels, and thus mutants defective in bleomycin transport are less likely to be isolated.

Survival Curve Determination. Survival after treatment with bleomycin, MMC, ICR-191, or EMS was determined by inoculating known numbers of cells into tissue culture dishes, incubating 2 to 4 h for attachment, and applying medium containing various concentrations of the agent. After a 16-h exposure, the medium was removed, the plates were rinsed, and fresh growth medium was added. For γ-ray survival, attached cells were irradiated with a 137Cs source at a dose rate of 56 rads/min. After 6 to 8 days of incubation, the number of surviving colonies was determined, survival curves were constructed, and D0, the dose which reduces cell survival to 37% of the untreated control, was determined using a replating and photography approach. As judged by the dose which reduces cell survival to 37% of the untreated control, these mutants are from 2.5- to 32-fold more sensitive to a 16-h bleomycin treatment than the parental cell, while for chronic bleomycin exposure, the increase in sensitivity was 5 to 58 times that of the parental cell. Four bleomycin-sensitive mutants had increased sensitivities to killing by γ-rays (2- to 3-fold), mitomycin C (2-fold), and ethyl methane sulfonate (4- to 5-fold), while six other mutants were resistant to these agents. Nine other bleomycin-sensitive mutants displayed a variable pattern of cross-sensitivities to these agents. Using the technique of alkaline elution, the relative frequency of single-strand DNA breaks introduced by varying concentrations of bleomycin was examined in one mutant and its parent cell. The elution profiles of both cells were similar, suggesting that the bleomycin sensitivity of this mutant is not due to a greater frequency of single-strand breaks introduced by bleomycin.

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The abbreviations used are: XP, xeroderma pigmentosum; CHO, Chinese hamster ovary; EMS, ethyl methane sulfonate; MMC, mitomycin C; AT, ataxia telangiectasia; D0, dose which reduces cell survival to 37% of the untreated control; PBS, phosphate-buffered saline.
values were determined (21). For survival to chronic bleomycin treatments, the cells were grown 8 to 10 days in medium containing various concentrations of bleomycin without replenishment, the number of colonies was determined, and survival curves were constructed as described above. Each point on the survival curve represents the average of at least 2 experimental determinations. \( D_{70} \) values were determined from survival curves. MMC and EMS were diluted into PBS buffer immediately prior to use. ICR-191 was dissolved in ethanol prior to use and stored at -20°C in aliquots. Bleomycin was dissolved in PBS buffer and stored at -20°C. MML and EMS were obtained from Sigma, ICR-191 was from Polysciences; and bleomycin (Benoxane) was a gift from Bristol Laboratories.

**Assay for DNA Damage.** The technique of alkaline elution was used to study the induction of single-strand breaks using the procedure as described by Kohn et al. (22) with modifications. DNA was uniformly labeled by growing cells 2 to 3 days in F-12 medium containing 0.01 \( \mu Ci/ml \) \([\text{methyl-3H}]\) thymidine (55 mCi/mmol; New England Nuclear) or 0.1 \( \mu Ci/ml \) \([\text{methyl-3H}]\) thymidine (20 Ci/mmol; New England Nuclear) for 2 to 3 days. A series of 25-cm\(^2\) flasks containing growth medium were inoculated with \( 1 \times 10^8 \) \( \mu \)labeled cells, incubated 2 h for attachment, and exposed for 16 h to varying concentrations of bleomycin. \(^3\)H-labeled cells were \( \gamma \)-irradiated (600 rads) at 0°C and kept on ice for use as an internal standard of DNA breakage. Cells were removed from flasks by gentle scraping with a rubber policeman into ice-cold PBS, and equal numbers (2 \( \times 10^7 \)) of \(^3\)C- and \(^3\)H-labeled cells were placed on 2-\( \mu m \) pore size polycarbonate filters (Millipore). Cells were washed with cold PBS and lysed on the filters by incubating in a solution containing 0.02 m trisodium EDTA, 2% sodium dodecyl sulfate, and 1 mg/ml proteinase K, pH 9.7, for 1 h. The lysis solution was collected, elution buffer (0.02 M EDTA plus tetrapropylammonium hydroxide to give pH 12.1) was gently added, and 5-ml fractions were collected at a flow rate of 0.056 ml/min. Total radioactivity in washes, eluted fractions, and on filters was determined as described previously (20).

**RESULTS**

**Mutant Isolation.** A photography and replica plating method was used to identify and isolate bleomycin-sensitive colonies. During the course of 10 separate mutant screens, approximately 150,000 colonies were analyzed for abnormal sensitivity to either a 16-h bleomycin treatment or 5-day bleomycin exposure, and 19 independent mutants were isolated. Survival curves of these 19 mutants for both 16 h and chronic treatment conditions are shown in Fig. 1. With the exception of BL-13, bleomycin-sensitive mutants isolated on the basis of a 16-h exposure (BL-1 to BL-13) had a two-slope survival curve: an initial steep slope and then a flattening of the curve at higher doses (Fig. 1).

Similar shaped curves are seen in wild-type cells at higher bleomycin concentrations. In contrast, mutants isolated using a 5-day treatment protocol (BL-14 to BL-19) have 16-h survival curves with significant shoulders (Fig. 1, I and K), while for chronic bleomycin treatment, survival curves of these mutants have either no shoulder or a greatly reduced one (Fig. 1, J and L).

The bleomycin-sensitive phenotype of these mutants appears to be stable. With the exception of BL-6, all mutants have been in continuous culture for at least 6 mo with no evidence of reversion. After recloning BL-6, the bleomycin-sensitive phenotype of this mutant also has been stable.

**Sensitivity to DNA-damaging Agents.** Table 1 lists doses of DNA-damaging agents needed to reduce cell survival to 37% \( (D_{70}) \). The value \( D_{70} \) represents the average dose required to kill a cell. For bleomycin, these values ranged from 0.19 to 2.4 \( \mu \)g/ml for a 16-h treatment and are 32- to 2.5-fold lower than the parental cell (6.1 \( \mu \)g/ml). For chronic bleomycin treatment, the \( D_{70} \) values ranged from 0.010 to 0.10 \( \mu \)g/ml and are 58- to 6-fold lower than that for the parental cell (0.58 \( \mu \)g/ml).

**Like bleomycin, \( \gamma \)-irradiation also produces single- and double-strand DNA breaks. Seven of the bleomycin-sensitive mutants (BL-3, -6, -7, -9, -15, -16, -19) had at least a 2- to 3-fold greater \( \gamma \)-ray sensitivity than the parental CHO cells, as judged by \( D_{70} \) values (Table 1), and three other bleomycin-sensitive mutants (BL-2, -12, -13) had at least a 3-fold greater \( \gamma \)-ray sensitivity than the parental CHO cells.

Sensitivity to the DNA cross-linking agent MMC was examined for all bleomycin-sensitive mutants. Eight bleomycin-sensitive mutants had at least a 2-fold greater sensitivity to MMC than the parental CHO cell with two mutants (BL-3, -8) having 4-fold increases in sensitivity (Table 1).
Two (BL-1, -4) of the 19 mutants showed a 2-fold increased sensitivity to the DNA intercalating agent ICR-191, and another mutant (BL-3) had a smaller (1.7) increase. Survival curves of MMC- and ICR-191-sensitive mutants were similar to those for γ-rays (data not shown).

Cross-sensitivity to the DNA alkylating agent EMS was also examined in the bleomycin-sensitive mutants. Eight bleomycin-sensitive mutants (BL-3, -4, -9, -12, -15, -16, -17, -19) had at least a 2-fold increase in EMS sensitivity, and three others (BL-1, -2, -7) had smaller (1.6- to 1.9-fold) but reproducible increases in sensitivities. Four of the nine mutants (BL-3, -9, -16, -19) had about a 4-fold increase in EMS sensitivity. Fig. 3 shows representative survival curves for two (BL-3, -19) of these mutants.

To better visualize the pattern of cross-sensitivities in these mutants, the mutants were grouped based on a 2-fold or greater increase in sensitivity to an agent (Table 2). Using this approach, the 19 bleomycin-sensitive mutants can be subdivided into eight groups (A to H): Group A contains 6 mutants that were not cross-sensitive to either γ-rays, MMC, ICR-191, or EMS; Group B contains 4 mutants sensitive to γ-rays, MMC, and EMS but not to ICR-191; Group C contains 2 mutants sensitive to MMC and EMS but not to γ-rays or ICR-191; Group D contains 2 mutants sensitive to MMC, ICR-191, and EMS, but not to γ-rays; Group E has 2 mutants sensitive to γ-rays and EMS but not MMC or ICR-191; Group F with one mutant sensitive to γ-rays but not to MMC, ICR-191, or EMS; Group G with one mutant sensitive to γ-rays and MMC, but not to ICR-191 or EMS; and Group H with one mutant sensitive to MMC but not to γ-rays, ICR-191, or EMS.

**Bleomycin-induced DNA Strand Breaks.** DNA strand breakage is considered to be a major reason for bleomycin cytotoxicity (23); however, 6 of the 19 bleomycin-sensitive mutants (Group A) are not cross-sensitive to any of the other types of DNA-damaging agents including γ-rays which also produce DNA breaks. One explanation is that, in these mutants, a relatively greater frequency of DNA breaks is produced by bleomycin, possibly due to increased permeability. Using the technique of alkaline elution, the relative frequency of single-strand DNA breaks induced by a 16-h bleomycin exposure was compared in the BL-10 mutant from Group A and its parental cell (Figs. 4 and 5). At 2 and 4 μg/ml bleomycin, no significant differences were observed in the relative frequency of single-strand DNA breaks produced in the 2 cells. In contrast, these bleomycin concentrations reduce survival in the BL-10 mutant to less than one cell per 100 and one per 10,000, respectively, whereas little reduction in survival occurs in the parental cell (75 and 50% survival). At higher bleomycin concentrations (8 μg/ml), which are out of the mutant's physiological range of survival, slightly more breaks appear to be introduced into the mutant cell than its parent. The above results suggest that increased yield of bleomycin-induced single-strand breaks does not account for the sensitivity in the BL-10 mutant.

**Table 2** Grouping of bleomycin-sensitive mutants by cross-sensitivity to DNA-damaging agents

<table>
<thead>
<tr>
<th>Group</th>
<th>Sensitivity to DNA-damaging agents</th>
<th>No. of mutants in group</th>
<th>Mutant strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>− − − −</td>
<td>6</td>
<td>BL-5, BL-10,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>BL-11, BL-13,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>BL-14, BL-18</td>
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<tr>
<td>B</td>
<td>+ + − +</td>
<td>4</td>
<td>BL-3, BL-9,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>BL-16, BL-19</td>
</tr>
<tr>
<td>C</td>
<td>− + − +</td>
<td>2</td>
<td>BL-17, BL-12</td>
</tr>
<tr>
<td>D</td>
<td>− + + +</td>
<td>2</td>
<td>BL-1, BL-4</td>
</tr>
<tr>
<td>E</td>
<td>+ − − +</td>
<td>2</td>
<td>BL-2, BL-5</td>
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<tr>
<td>F</td>
<td>+ + − +</td>
<td>1</td>
<td>BL-6</td>
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<tr>
<td>G</td>
<td>+ + − +</td>
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</tr>
<tr>
<td>H</td>
<td>− − − −</td>
<td>1</td>
<td>BL-8</td>
</tr>
</tbody>
</table>
equal numbers of "(-labeled cells treated with bleomycin, and the cell mixture subjected to alkaline elution. Symbols indicate bleomycin concentrations.

bleomycin for 16 h. 3H-labeled cells were ^gamma^-irradiated with 600 rads, added to parental (I) and BL-10 cells (B) to various concentrations of control. 3H-DNA retained was determined for 3 independent experiments and constructed as in Fig. 4, and the percentage of 3H-DNA was retained at 50% of the averages plotted. Bars, SD.

BL-10 (O) by various concentrations of bleomycin. Elution profiles were constructed as in Fig. 4, and the percentage of 3C-DNA was retained at 50% of control. The patterns of cross-sensitivity in one of these mutants indicate bleomycin concentrations.

DISCUSSION

In this paper, we describe the isolation of 19 Chinese hamster mutants which are sensitive to the antitumor drug bleomycin. Studies on isolated DNA indicate that bleomycin produces a variety of DNA lesions including single- and double-strand breaks (23), alkaline-sensitive sites (24), the release of free bases (25), noncovalent intermolecular cross-links (26), and DNA degradation (27). Thus, mutants sensitive to this agent could be defective in a variety of DNA repair pathways. Consistent with this possibility is the finding that these bleomycin-sensitive mutants can be divided into eight groups based on their pattern of cross-sensitivity to four different types of DNA-damaging agents: a cross-linking agent (MMC); an alkylating agent (EMS); an intercalating agent (ICR-191); and a DNA strand-breaking agent (gamma-rays). Further, initial cell fusions between mutants BL-10, BL-12, BL-7, and BL-8 from Groups A, C, G, and H produced hybrids with wild-type bleomycin sensitivity, indicating that these mutants are in separate bleomycin complementation groups.4

The patterns of cross-sensitivity in one of these mutants (Group B) have similarities to the EM-9 mutant isolated by Thompson and coworkers (28). The EM-9 mutant is defective in the repair of single-strand DNA breaks and, like the mutants in Group B, is also sensitive to EMS and gamma-irradiation. There are, however, some differences between these mutants. EM-9 is 2 to 3 times more sensitive to EMS than the Group B mutants but is not sensitive to MMC and only has partial sensitivity to bleomycin (29). Likewise, mutants in Group E are also sensitive to both EMS and gamma-irradiation but not to MMC, although EMS sensitivity in this group is generally less than in Group B.

One major group (A) of 6 bleomycin-sensitive mutants is not sensitive to any of the other 4 types of DNA-damaging agents. These mutants might have an increased frequency of DNA damage per dose of bleomycin resulting from a selective alteration in membrane permeability (or transport) that increases the intercellular bleomycin concentration without affecting permeability of the other DNA-damaging agents. Alternatively, the intercellular concentration of bleomycin could be higher in these mutants because of a defect in bleomycin hydrolase, which degrades bleomycin (23). It is also possible that a metabolic change in the cell increases the effectiveness of bleomycin in producing the lethal lesion. This change could be in oxygen metabolism since in vitro studies indicate that this molecule is required by bleomycin for DNA strand breakage (23). However, for mutant BL-10 from Group A, the relative frequency of single-strand DNA breaks measured by alkaline elution was not significantly different in the mutant and parent cell. Thus, mutant BL-10 may be defective in the repair of a lesion induced uniquely by bleomycin and not produced by any of the other DNA-damaging agents tested.

Recently, Robson et al. (30) have reported the isolation of two bleomycin-sensitive mutants and have examined their sensitivity to a range of DNA-damaging agents similar to those used in the present study. Those mutants have a different set of cross-sensitivities than the mutants described in this report and may represent additional genetic classes of bleomycin-sensitive mutants.

Although many in vitro studies suggest that bleomycin exerts its cytotoxic effect through DNA damage, the in vivo mechanism for the cytotoxic action of bleomycin is not well understood (23). A study of the biochemical basis for the increased bleomycin sensitivity of these mutants should provide important information concerning the mechanism for bleomycin cytotoxicity. Such information could be extremely valuable in developing new and more efficient antitumor agents. Bleomycin-sensitive mutants should also be useful as recipient cells in DNA transfection experiments using human DNA. Such approaches offer the prospect of isolating human genes involved in bleomycin resistance.

In conclusion, we have isolated 19 significantly bleomycin-sensitive mutants in the Chinese hamster ovary cell and characterized them on the basis of their cross-sensitivity to other DNA-damaging agents. For at least one mutant, bleomycin sensitivity is not due to increased frequency of single-strand DNA breaks. We believe these mutants will be extremely useful in elucidating the molecular mechanism for the in vivo toxicity of this agent and the role DNA repair may play in this process.

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