Pulse Cytophotometric Analysis of Cell Cycle Perturbation with Bleomycin in Vitro

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SUMMARY

The kinetic response of a human lymphoma cell line to bleomycin has been analyzed, using an ICP-11 pulse cytophotometer. Bleomycin induced a delay of the cell-cycle traverse in G2 phase, the extent and recovery of which depended on drug concentration, exposure time, and cell cycle stage where treatment was applied. Different phase sensitivity for lethal damage (G2 phase) and kinetic response (early S phase) were documented. Recovery from G2 block did not predict for unimpaired reproductive capacity.

INTRODUCTION

The complex glycopeptide BLM2 has received rapid and extensive clinical trials in a variety of human cancers, both as a single agent and in combination (5, 6, 12, 17, 19). The current basis for combination chemotherapy has been empirical, based on antitumor activity and toxicity. Recently, some investigators have included kinetic concepts derived from in vitro studies in the design of clinical protocols (4, 16, 20, 25). These kinetic concepts are based on studies evaluating the effects on cell cycle progression that suggest that BLM may behave as a synchronizing agent. For BLM, most authors report an arrest in G2 phase (1, 3, 7, 14, 18, 21, 23, 24, 26), although delay in phases other than G2 phase has been observed (26). However, there is some controversy about the phase of the cell cycle, where exposure of cells to BLM will subsequently produce maximum G2 block (3, 14).

The mode of action by which BLM induces both lethal and cytokinetic effects remains to be elucidated. It has been reported that BLM attacks DNA, producing strand breaks (15, 22); however, the correlation among DNA strand scission, G2 delay, and lethality is unknown. Survival studies have shown that M- and G2-phase cells are the most sensitive to the lethal effects of BLM, as assessed by loss of reproductive capacity (3, 8). Using the premature chromosome condensation technique, Hittleman and Rao (14) have demonstrated that S-phase cells suffer similar or even slightly greater chromosomal damage from BLM treatment than G2-phase cells. However, a correlation between chromosome damage and cell lethality has not yet been established.

We have been interested in whether there was a relation between the cytokinetic and lethal effects of BLM. Therefore, we investigated the cell cycle progression of a cultured human lymphoma cell line perturbed with various concentrations of BLM for different exposure times, using pulse-labeling and DNA histogram techniques. Our data showed a concentration- and exposure time-dependent, partially reversible G2 arrest. The delay in G2 was most effective for early S-phase cells. No correlation between the cytokinetic effects, measured by PCP (2), and lethal effects, assessed by colony-forming capacity (8, 10), was demonstrated.

MATERIALS AND METHODS

Cell Line

A human immunoglobulin-producing lymphoma cell line (T1 cells) was grown as a monolayer in Ham's F-10 medium, supplemented by 20% fetal calf serum, glutamine, antibiotics, and vitamins (11). Under these conditions, the kinetic parameters are: generation time, $T_T = 27$ hr, with $T_{G1} = 3.5$ hr; $T_S = 13.5$ hr; $T_{G2} = 10$ hr; and $T_{M} = 0.43$, as determined by the percentage of labeled mitoses technique.

For experiments using asynchronous cells, approximately $5 \times 10^6$ cells were plated into 60-mm Petri dishes with 3 ml of medium and incubated for 48 hr to achieve exponential growth; at this time, treatment with various concentrations of BLM, extended for differing periods of time, was started.

For experiments involving synchronized cells, a TdR block (3 mM for 24 hr) was applied to exponentially growing cultures, giving a synchrony yield of 85 to 90% cells in S phase as determined by tritiated deoxycytidine pulse labeling (9) and DNA histograms (2). Tritiated deoxycytidine was used instead of $[^3H]TdR$ to bypass the block of exogenous TdR uptake induced by the synchronizing agent (11). All experiments were carried out in duplicate.

Drug

BLM (Blenoxane, 15 mg/vial; Bristol Laboratories, Syracuse, N. Y.) was dissolved in 5 ml of 0.9% NaCl solution, and further diluted with medium immediately prior to the experiment.

Cytokinetic Analysis

Li and Mi. For radioautography, cytocentrifuge preparations were fixed with 5% trichloroacetic acid and 95% meth-
anol and were dipped in Kodak Nuclear Track Emulsion, type NTB 2(diluted 1:3 with distilled water). After developing (Kodak D-19) and fixing (Kodak fixer), cells were stained with May-Grünewald-Giemsa. Two hundred cells were scored for determination of the LI (>5 grains/nucleus) and 3000 cells for MI.

DNA Histograms. Samples were centrifuged at 2000 rpm for 1 min. The cells were resuspended in 0.5 ml of 0.9% NaCl solution and fixed by adding 1.2 ml of absolute ethanol. The ethanol-fixed cells were stained with mithramycin (50 μg/ml) and analyzed by means of an ICP-11 pulse cytophotometer, as described before (2). Cells (50,000 to 100,000) were measured for each DNA histogram. The standard deviation for the compartment size of G1, G2, and S fractions did not exceed 2.5%.

RESULTS

Asynchronous Cells. Chart 1 shows a DNA histogram of an asynchronous exponentially growing population of T1 cells (48 hr after plating stock cultures). The average distribution of cells in each phase is G1 = 50%, S = 35%, and (G2 + M) = 15%.

Continuous incubation of these cells with BLM at concentrations of 5, 10, 25, 50, 100, and 500 μg/ml showed a concentration-dependent steady accumulation of cells in the (G2 + M) fraction, at the expense of both the G1 and S-phase compartments, reaching 60% of the total population (Chart 2); after 24 hr, there was no further change in the distribution of cells in the different histogram fractions for any concentration. The minimum values for G1 and S at a concentration of 100 μg BLM per ml are 25 and 15%, respectively. Chart 3 shows the accumulation of (G2 + M) cells, as measured by PCP, and the corresponding MI as a function of incubation time. The MI of treated cells drops drastically after 4 hr, indicating that the compartment measured by PCP consists virtually of G2 cells. In these experiments, we noted a concentration-dependent dissociation between the percentage of cells in S phase measured by PCP and that measured by conventional LI ([3H]TdR); this occurred after 24- to 28-hr incubation with BLM for concentrations greater than 25 μg/ml and reached a maximum after 2 hr to 3 hr (Chart 4). This dissociation was maintained over the ensuing period of the experiment.

In other experiments, T1 cells were incubated with in-
Increasing concentrations of BLM (25, 50, and 100 μg/ml) for 12 hr. The drug was decanted, and the cells were washed twice with Hanks' balanced salt solution and reincubated with fresh medium. Cytokinetic effects were followed by PCP for the next 60 hr. Chart 5 shows a moderate, dose-dependent increase in the G2 compartment which peaks 12 hr after drug removal, followed by a decrease at 36 hr. The MI corresponding to the treated cells (Chart 6) shows a 5- to 7-fold increase after removing the drug over that of cells still under the influence of the drug. The timing of this increase appears dose related; the peak corresponding to cells treated with 100 μg/ml occurs 12 hr after that of cells treated with 25 and 50 μg/ml.

Asynchronous T4 cells were incubated with 100 μg of BLM per ml for 3, 6, 9, and 12 hr. The drug was decanted, the cells were washed twice and reincubated with fresh medium. Compartment distribution was followed for the ensuing 60 hr. Chart 7 shows an exposure time-dependent accumulation in G2, peaking at 12 hr after drug removal, with a subsequent decline in the percentage of G2 cells. This increment of the G2 compartment occurs primarily at the expense of S-phase cells (which are reduced 3- to 4-fold of control), while a minor contribution of G1-phase cells (<2-fold) occurs only after incubations longer than 9 hr. In these experiments, there was no dissociation of S-phase cells measured by PCP and [3H]TdR LI.

Chart 8 shows the maximum percentage of G2-phase cells accumulated by various concentrations and time incubations of BLM, regardless of the time point at which this percentage was obtained.

Synchronized Cells. Incubation of T4 cells with BLM (100 μg/ml) for 1 hr in different phases of the cell cycle resulted in a transitory, small increase in the subsequent G2 fraction, mainly at the expense of cells in G1 (Table 1). This effect, noted 19 hr after TdR release, was somewhat more pro-

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**Chart 4.** Effect of BLM concentration on the degree of dissociation of S-phase percentage (PCP) and LI percentage ([3H]TdR) after continuous treatment of asynchronous T4 cells for 32 hr.

**Chart 5.** Effect of a 12-hr continuous incubation with BLM (25, 50, and 100 μg/ml) on the distribution of asynchronous T4 cells in the various phases of the cell cycle (PCP).

**Chart 6.** MI of asynchronous T4 cells treated with BLM for 12 hr.

**Chart 7.** Effect of treatment with 100 μg BLM per ml (3-, 6-, 9-, and 12-hr incubation) on the distribution of asynchronous T4 cells in the various phases of the cell cycle (PCP).
nounced when BLM was dispensed in late S or early G2. By 80 hr after TdR release, the distribution of all treated cells returned to control values. Cells treated in G1 showed no alteration in the subsequent distribution in the various compartments of the cell cycle with respect to control cells.

Table 2 gives the distribution of T1 cells for various synchronized populations continuously incubated with 100 μg of BLM per ml, measured 15 hr after release of the TdR block, that is at the time of late G2 for control cells. At this time, the proportion of G2 cells of treated populations was similar to that of control cells, regardless of the time at which drug perturbation was started. When the distribution of cells was measured 15 hr after commencing continuous drug incubation of different synchronized populations, a pattern of decreasing G2 accumulation effect (accompanied by an increase of the G1 fraction) from treatment initiated in early S to treatment begun in late G2 was observed (Table 3). In every instance, the proportion of cells accumulated in G2 was maintained over the length of 1 generation time after TdR release (Table 4).

In other experiments, synchronized cells were continuously exposed to 100 μg of BLM per ml. Treatment was initiated in early S, mid-S, late S, early G2, and late G2. Twenty-four hr after TdR release, the drug was decanted, the cells washed twice with Hanks’ balanced salt solution, and reincubated with fresh medium. The distribution of cells in the various compartments was measured at this time.

Table 2

<table>
<thead>
<tr>
<th>Phase of BLM addition</th>
<th>G1</th>
<th>S</th>
<th>G2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>19</td>
<td>10</td>
<td>71</td>
</tr>
<tr>
<td>Early S</td>
<td>15</td>
<td>7</td>
<td>16</td>
</tr>
<tr>
<td>Mid-S</td>
<td>9</td>
<td>2</td>
<td>84</td>
</tr>
<tr>
<td>Late S</td>
<td>8</td>
<td>6</td>
<td>30</td>
</tr>
<tr>
<td>Early G2</td>
<td>6</td>
<td>12</td>
<td>13</td>
</tr>
</tbody>
</table>

* A, percentage of cells at time of drug addition; B, percentage of cells 15 hr after TdR release (late G2).

(0 hr) and at regular intervals thereafter for the subsequent 48 hr. Again, treatment of early- and mid-S-phase cells resulted in the maximum accumulation in G2 (Chart 9) with values above that of control when measured at the time of drug removal. After reincubation with fresh medium, only a few cells that had been treated beginning in early and mid-S phase progressed from G2 into G1. However, large numbers of cells accumulated in G2 by treatment initiated in late S and G2 rapidly moved into G1 (within 3 hr) in the subsequent drug-free incubation interval. In this case, the proportion of cells remaining in G2 for the next 48 hr was directly dependent on the time at which drug treatment had been initiated, the largest fraction corresponding to cells treated in late S.

G1-phase populations synchronized by a previous TdR block were exposed to continuous incubation with 100 μg of BLM per ml for 24 hr. Then, these cells were washed and reincubated with fresh medium. Compartment distribution was measured for the ensuing 24 hr. A relatively small accumulation of cells in G2 (30%) was observed at the end of drug exposure, which remained unchanged over the next 24 hr (Chart 9).

DISCUSSION

Our studies with PCP show that BLM accumulates cells in (G2 + M), without affecting the transit through S phase, even during continuous exposure. Since the MI for the treated

![Chart 8. Maximum G2 accumulation after treatment of asynchronous T1 cells with BLM for various concentrations (25, 50, and 100 μg/ml) and various exposure times (3, 6, 12, 24, and 48 hr).](image-url)
Table 3

Effect of continuous incubation with BLM (100 µg/ml) on synchronized T, cells observed 15 hr after drug addition

<table>
<thead>
<tr>
<th>Phase of BLM addition</th>
<th>Control</th>
<th>BLM</th>
<th>Control</th>
<th>BLM</th>
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<tbody>
<tr>
<td>Early S</td>
<td>30</td>
<td>5</td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td>Mid-S</td>
<td>72</td>
<td>9</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Late S</td>
<td>89</td>
<td>31</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>Early G₂</td>
<td>82</td>
<td>45</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>Late G₂</td>
<td>82</td>
<td>62</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

a Measured 15 hr after initiation of BLM treatment on duplicate cultures.
b Expressed as percentage of the total population.

Table 4

Effect of continuous incubation with BLM (100 µg/ml) on synchronized T, cells observed 36 hr after TdR release

<table>
<thead>
<tr>
<th>Phase of BLM addition</th>
<th>G₁</th>
<th>S</th>
<th>G₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>71</td>
<td>14</td>
<td>15</td>
</tr>
<tr>
<td>Very early S</td>
<td>10</td>
<td>10</td>
<td>81</td>
</tr>
<tr>
<td>Early S</td>
<td>19</td>
<td>14</td>
<td>67</td>
</tr>
<tr>
<td>Mid-S</td>
<td>23</td>
<td>14</td>
<td>63</td>
</tr>
<tr>
<td>Late S</td>
<td>32</td>
<td>17</td>
<td>51</td>
</tr>
<tr>
<td>Early G₂</td>
<td>49</td>
<td>10</td>
<td>41</td>
</tr>
</tbody>
</table>

a Expressed as percentage of the total population.

populations is smaller than control values, the BLM-induced increase in the (G₂ + M) fraction of the DNA histogram is due to an accumulation of cells primarily in G₂. This means that BLM delays and blocks cell cycle progression in G₂, confirming, in a human cell line, results published by a number of other investigators (1, 3, 7, 14, 18, 21, 23, 24, 26). On asynchronous cells, this effect is concentration and time dependent for concentrations lower than 100 µg/ml and incubation intervals shorter than 48 hr (Chart 8). Experiments on synchronized cells demonstrate phase sensitivity with maximum G₂ accumulation (85 to 90%) for cells, the treatment of which began in early S phase. This pronounced age dependency of G₂ delay has not been reported for BLM. Using the technique of premature chromosomal condensation, Hittleman and Rao (14) found considerable damage in CHO cells treated in S phase. Cells are able to recover partially from the BLM-induced G₂ block. The degree of recovery is related to the position of the cells in the cycle at the time of initiation of drug treatment, in a fashion similar to that observed with the efficacy of BLM in inducing G₂ accumulation. Thus, the transit block in G₂ phase of cells that are treated in G₂ is only partial. Under these conditions, about 50% of the blocked G₂ cells can escape and undergo mitosis resulting in a subsequent increase of the G₁ compartment.

The dissociation of LI and S-phase cells measured by PCP during continuous incubation with BLM indicates an increased incidence of "U"-cells (13) occurring after 24 hr of continuous incubation with BLM. However, no delay in S-phase transit was observed (Table 2). This suggests that cells escaping the G₂ block, possibly those that were in late S and G₂ at initiation of drug incubation, constitute the majority of U-cells. Two possible mechanisms may be responsible for this phenomenon: (a) cells synthesize DNA but are unable to incorporate [³H]TdR; or (b) cells are blocked in some interval of S phase with no further DNA synthesis taking place, but they appear as S-phase cells by PCP. Our data do not permit identification of which mode is operating.

Comparing our data on the effects induced by BLM on cell cycle perturbation and its lethal effects on this cell line (8, 9), we find different phase sensitivities for the institution of maximum G₂ delay (early S phase) and for maximum cell kill (G₂ phase). However, in survival experiments, BLM exposure time was limited to 1 hr, which originated only a 1-log cell kill with 100 µg/ml. In perturbation experiments, this short incubation time caused only a small, reversible, cell cycle-independent accumulation of cells in G₂ (Table 1). Under conditions resulting in an effective and reversible G₂ block (i.e., 50 µg/ml for 24 hr = 45% cells accumulated in
G₂, the lethal effects were more pronounced (i.e., 3-log decades of killing). Hence, it is noteworthy to indicate that recovery from cycle-progression delay does not imply unimpaired reproductive capacity. Thus, in spite of some mechanism enabling cells to overcome the G₂ block, the number of subsequent cell divisions, as assessed by colony formation, is considerably reduced, which indicates that, at least in the case of BLM, cytokinetic effects do not predict lethal effectiveness.

Our in vitro results on perturbation of the cell cycle still favor BLM as an effective phase-sensitive synchronizing agent. Although BLM induces a considerable degree of irreversible G₂ block (with subsequent cell death), cells able to escape this block will emerge synchronously. A fraction of these cells (or their progeny) will subsequently die from the inherited damage; but those cells that escape the G₂ block with intact reproductive capacity will be amenable to complete sterilization by the scheduling of a 2nd-phase sensitive drug at full cytocidal dose used at an optimum time interval. There are encouraging preliminary data on patients with head and neck cancer treated sequentially with BLM and X-radiation using such a kinetic rationale based on continuous PCP monitoring (20, 25).

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