The Response of Synchronized Human Lymphoma Cells to Bleomycin and 1,3-Bis(2-chloroethyl)-1-nitrosourea

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SUMMARY

Synchronized human lymphoma cells treated with bleomycin exhibited the greatest sensitivity in G2 phase. Dose-response curves presented biphasic characteristics indicating the presence of sensitive and less sensitive cells in all stages of the cell cycle. Synchronized cells incubated with 1,3-bis(2-chloroethyl)-1-nitrosourea also showed the greatest sensitivity in G2 phase. A threshold-type dose-response survival curve was elicited in all phases of the cell cycle suggesting that damage must be accumulated before cell killing is expressed even in the sensitive stages.

INTRODUCTION

We have recently reported the effect of 2 currently used chemotherapeutic drugs, BLEO and BCNU, on asynchronous human lymphoma cells cultured in vitro (14). BLEO treatment generated a biphasic survival curve suggesting the possibility of sensitive and less sensitive cells. Treatment with BCNU originated a threshold-type survival curve similar to that observed after X-ray treatment. The present report extends these studies into the investigation of the effects of these 2 drugs on synchronized cells in order to evaluate possible cell cycle stage sensitivities.

MATERIALS AND METHODS

A human immunoglobulin-producing cell line (T1 cells) derived from the tumorous lymph node of a patient with lymphocytic lymphoma was used (28). The cells carry a tumor-associated antigen common to lymphomas (29). Monolayer cultures were maintained in Ham's F-10 medium supplemented with 20% fetal calf serum, vitamins, glutamine, and antibiotics. Under these conditions, the average cell cycle time was 27 hr. The pre-DNA synthesis period (G1) was 3.5 hr, the DNA synthesis period (S) was 13.5 hr, and the post-DNA synthesis period (G2) was 10 hr (11). Cell synchronization was carried out as previously described (13). Briefly, synchronized S-phase cells were obtained by a single treatment with 3 mM TdR. The degree of synchrony was monitored in replicate cultures by 30-min pulse labeling with 1 μCi of TdR-3H per ml, to determine the percentage of cells in S phase, and by scoring the MI. At the end of the synchrony procedure, 90% of the cells were in S phase and moved synchronously into G2 (80%), estimated from the LI and the MI. After a mitotic peak of 10 to 12% (the MI of asynchronous T1 cells ranges from 0.6 to 1.8), the cells were still partially synchronized in G1 (70 to 80%). Ten to 12 hr after the mitotic peak the LI began to rise again although achieving a much lower peak (50%) than the initially synchronized cells in S. To increase the yield of G1-phase cells, a single block of TdR of 24 hr duration was followed by mitotic selection at the time calculated for the arrival of the cells at the end of G2. This technique provided up to 93% cells in mitosis, which cells promptly entered into G1 phase after incubation at 37°. These procedures did not significantly alter the plating efficiency of T1 cells although they produced a shortening of S and G2 phases and an elongation of G1 phase. In all radioautography procedures a 50% solution of Ilford K5 emulsion in distilled water was used. Labeled cells were identified by the presence of 5 or more grains overlying the nucleus. Synchronized T1 cells were exposed for 1 hr to a single concentration of freshly prepared drug every 2 hr during the full length of the cell cycle. In other experiments, a survival response to increasing concentrations of drug was determined at selected points of the cell cycle. Cells incubated for 6 hr in fresh medium, after removal of the synchronizing agent, were selected as representative of mid-S-phase cells. Cells incubated for 15 hr were considered G1-phase population and finally G1 populations were produced by incubation for 19 hr in normal conditions (3 to 4 hr after the mitotic peak) or 3 hr after mitotic selection procedures.

After treatment, the cells were washed twice with fresh medium, harvested as a single cell suspension (15), and counted with the aid of an electronic particle counter. Appropriately cell concentrations were seeded in Petri dishes so that 50 to 100 colonies would appear after a 3-week incubation period. The colonies were rinsed with 0.9% NaCl solution, fixed, stained with crystal violet, and examined under a stereomicroscope (10). Cell survival was defined as the capacity of single cells to generate colonies of 50 or more cells. Controls (synchronized cells not treated with drug) were run in parallel for each experiment. Percentage of survival was calculated in reference to controls with the
aid of a Sigma 5 XDS computer. Each experiment was repeated at least twice with 3 dose or time experimental points and 6 control points each.

RESULTS

Synchronized T<sub>1</sub> cells exposed to a single dose of BLEO (50 or 100 µg/ml) for 1 hr at regular intervals during the cell cycle demonstrated fluctuations in survival (Chart 1). The relatively most resistant population was found in mid-S while G<sub>1</sub> and G<sub>2</sub> cells, especially late G<sub>2</sub> cells, exhibited the greatest decrease in survival. There was a difference of about 1 log decade between the more resistant to the more sensitive population. Dose-response survival curves were obtained by exposing synchronized cells to increasing concentrations of BLEO at selected points of the cell cycle (Chart 2). In all 3 stages (mid-S, G<sub>2</sub>, and G<sub>1</sub>) biphasic survival curves were obtained similar to that encountered in asynchronous T<sub>1</sub> cells treated with BLEO for 1 hr (14). The proportions of sensitive cells and the corresponding D<sub>0</sub>'s varied markedly among the synchronized populations (Table 1). It can be noted that both G<sub>1</sub> and G<sub>2</sub> cells have a similar proportion of sensitive populations with almost equal D<sub>0</sub>'s. However, the D<sub>0</sub> of the relatively more resistant cells present in G<sub>2</sub> is about one-half of the D<sub>0</sub> of both mid-S and G<sub>1</sub> cells.

Synchronized T<sub>1</sub> cells exposed to a single concentration of BCNU (50 µg/ml) for 1 hr at regular intervals during the cell cycle revealed a moderate fluctuation in survival along the cell cycle (Chart 3). S-phase cells were relatively resistant while G<sub>2</sub> phase cells appeared more sensitive. Although G<sub>1</sub> cells appeared as resistant as S-phase cells, their sensitivity increased as they approached S; the G<sub>1</sub>-S border showed an intermediate sensitivity. Dose-response survivals obtained in mid-S, G<sub>2</sub>, and G<sub>1</sub> demonstrated shoulder-type survival curves similar to that obtained in an asynchronous population (14) (Chart 4). There were considerable variations in the magnitudes of both the shoulder region and the D<sub>0</sub> of the various synchronized populations (Table 2). It appears that although G<sub>2</sub> cells have a greater capacity to absorb damage without expressing a lethal effect (n = 15) once this capacity is overcome the cells are sterilized in a highly efficient manner on a per unit drug basis (D<sub>0</sub> = 8 µg/ml). The converse holds true for both G<sub>1</sub> and S-phase cells.

DISCUSSION

Both BCNU and BLEO are currently in use in a variety of antineoplastic chemotherapy protocols (7, 8, 23, 30, 31). These drugs appear promising in the control of tumors of the lymphoreticular system (17, 18, 21). Our in vitro studies are considered representative of the survival of in vivo neoplastic cells once the drug reaches the malignant target cells, thereby providing fundamental data which may be used to develop improved clinical chemotherapeutic protocols.

The killing effect of BLEO on synchronized human lymphoma cells is similar to that reported for Chinese hamster cells (2) in that the most sensitive stage is G<sub>2</sub>. However, this effect is not as marked as that reported for this cell line and G<sub>1</sub> cells also appear very sensitive. Although mitotic cells may be as sensitive as G<sub>2</sub> cells (2), the effect of BLEO on this stage was not studied with T<sub>1</sub> cells. The lethal mode of action of BLEO is unknown. BLEO inhibits DNA synthesis (24), produces DNA strand scissions (25), and also causes cell progression delay in G2 (1, 19, 27). Progression delay may result from the interference of BLEO with the synthesis or function of a “division-specific protein” (2). Nagatsu et al. (20) have suggested that inhibition of tumor growth results from the effect of the drug on cell progression and not from its inhibition of DNA synthesis. We have carried out preliminary studies on the effect of BLEO on the progression of T<sub>1</sub> cells that suggest that the drug may interfere with the transit of G<sub>2</sub> cells into mitosis in a fashion similar to that of other mammalian cells. If these preliminary data are confirmed, they would support the contention that BLEO kills cells mostly by interfering with the transit of G<sub>2</sub> cells and not by inhibiting DNA synthesis (9) since G<sub>2</sub> is also the stage most sensitive to the lethal effect of the drug. The effects of cell progres-
sion blocking and cell killing would be time-, and perhaps, dose-dependent, analogous to the mechanism of unbalanced growth obtained with DNA synthesis inhibitor drugs (16). Low concentrations of BLEO given for short periods would yield a partial block in G₂ while higher concentrations for longer time intervals would produce cellular death.

Asynchronous Tₘ cells showed a biphasic survival curve when exposed to BLEO for 1 hr (14). Biphasic curves were also elicited from synchronized cells, in all stages of the cell cycle. However, the proportion of sensitive and less sensitive cells and the respective D₀ varied independently at each stage suggesting that these 2 populations have independent sensitivities during the cell cycle. Table 1 shows that the D₀'s of the sensitive cells of G₁ and G₂ populations are very similar and about 2-fold less than the D₀ of sensitive S-phase cells. However, while the D₀ of resistant S-phase cells is about the same as the D₀ of resistant G₁ cells, the corresponding D₀ of G₂ cells is just one-half of the former D₀'s. Such values could explain the increased sensitivity of G₂ cells by an increment in overall cell killing due to a higher proportion of killed resistant cells. In experiments involving synchronized cells there is always some degree of uncertainty about the grade of synchronization achieved by the techniques used. Furthermore, synchronization is an ephemeral attainment since soon, after normal progression is allowed, cells rapidly enter into asynchronous growth as a result of the random rates of cellular growth (13). However, in our system, and judging by the temporal markers used (LI and MI), there was a substantial accumulation of phased cells in the different stages of the cell cycle.

<table>
<thead>
<tr>
<th>Stage</th>
<th>D₀ (μg/ml)</th>
<th>% sensitive population</th>
<th>D₀ₛ (μg/ml)</th>
<th>% survivors after 100 μg/ml exposure (1 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mid-S</td>
<td>11</td>
<td>56</td>
<td>60</td>
<td>11</td>
</tr>
<tr>
<td>G₂</td>
<td>4</td>
<td>75</td>
<td>32</td>
<td>1</td>
</tr>
<tr>
<td>G₁</td>
<td>5</td>
<td>85</td>
<td>65</td>
<td>4</td>
</tr>
<tr>
<td>Asynchronous</td>
<td>17</td>
<td>82</td>
<td>140</td>
<td>8</td>
</tr>
</tbody>
</table>

*a D₀ₛ, inverse of the secondary slope.
BLEO and BCNU on Synchronized Lymphoma Cells

20 40 60 80 100
CONCENTRATION OF BCNU (µg/ml)

Chart 4. The effect of increasing concentrations of BCNU on synchronized T, cells.

Therefore, we believe that biphasic curves elicited in all stages of the cell cycle are the result of 2 separate populations of cells in the T, cell line and not due to contaminations of population in different stages of the cycle. This is substantiated by the fact that both G2 and G1 cells (the latter obtained by 2 separate techniques) demonstrated biphasic curves. If these cells would have been contaminated with each other in our experiments (late G2 or early G1, when almost no labeled cells were noted by radioautographic techniques), a continuous exponential curve would have been obtained, the slope of which being the summation of G2 and G1 cell slopes. Yet, in either case we obtained biphasic curves. Similar biphasic curves were obtained by Barranco and Humphrey (2) when they studied Chinese hamster cells where the degree of synchrony was superior to the one obtained with our human cell line.

Synchronized T1 cells treated with BCNU revealed some fluctuation in their survival, depending on the stage of the cell cycle. The most sensitive cells were those located in G2. Our results differ from those of Bhuyan et al. (6), who indicated that the G1-S boundary and to a lesser degree the S-G2 boundary were the most sensitive points of the cell cycle. Although we observed some sensitivity in the G1-S boundary it was quantitatively less than that observed with mid-G2 cells. Barranco and Humphrey (1) have reported that BCNU kills Chinese hamster cells most efficiently in S phase while preventing cell progression in all phases of the cell cycle. However, these investigators did not carry out cell survival experiments in G2 phase and it is possible that Chinese hamster cells may exhibit an even greater sensitivity in G2 than that observed in mid-S. We also noted a moderate dip in the survival curve in mid-S at a point roughly corresponding to the stage of greatest sensitivity in Chinese hamster ovary cells. Nevertheless, by extending the survival experiments into the G2 stage we obtained a greater degree of cell killing.

BCNU has been considered an alkylating agent (30) and to exert a lethal effect by attacking guanine in position 7 (26). It has also been postulated (1) that BCNU may interfere with the synthesis of a division-specific protein, thus causing G2 progression delay. Therefore, it is conceivable that BCNU may kill certain cells more efficiently in G2 by the latter mechanism than by its action as alkylating agent. These differences in mode of action may be expressed in various proportions with different cell lines or even with different strains obtained from a seemingly homogeneous source (5). These killing mechanisms can only be effective in dividing cells. However, Barranco et al. (4) have shown that BCNU may kill resting phase cells more efficiently than actively dividing ones, which suggests a 3rd, as yet unknown, killing modality for this drug.

Dose-response survival curves obtained at mid-S, G1, and G2 all showed the presence of a shoulder region suggesting that damage must be accumulated in order to express cell lethality. However, there were marked differences in the magnitudes of such shoulders as well as in the killing efficiencies (D0) observed in the exponential parts of the curves depending on the stage of the cell cycle. G2 cells had the largest shoulder (n = 15) and the lowest D0 (D0 = 8). S- and G1-phase cells presented short shoulders (n = 2 and n = 3), while the D0 was relatively large (30 and 20, respectively). This indicates that while the target of action of BCNU in G2 cells can absorb a considerable amount of damage, once this capacity is exhausted the target structure is extremely sensitive to the killing effect of the drug. The opposite situation would occur with G1- and S-phase cells; the capacity to absorb damage without expressing cell lethality is relatively decreased but target resistance is increased.

Sinclair and Morton (22) have indicated that a threshold-type survival curve obtained with asynchronous cells after

<table>
<thead>
<tr>
<th>Stage</th>
<th>n</th>
<th>D0 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>S</td>
<td>2</td>
<td>30</td>
</tr>
<tr>
<td>G2</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>Asynchronous</td>
<td>3</td>
<td>17</td>
</tr>
</tbody>
</table>

Table 2
Relative sensitivities of synchronized T1 cells to BCNU

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X-radiation represent the summation of the survival curves resulting from the exposure of synchronized cells at various stages of the cell cycle. This has also been observed with X-radiated T₄ cells where the survival curve obtained for asynchronous cells closely approached that obtained from the summation of the synchronized components (11). Using a similar computation, the survival curve resulting from the summation of the survival curves of synchronized T₄ cells treated with BCNU yields an n of 4 and a D₀ of 20, which is very close to that obtained with asynchronous cells (n = 3 and D₀ = 17 μg/ml).

Our data indicate that both BLEO and BCNU, although not to be considered G₂-specific agents, will kill cells more efficiently in this stage. Thus they could be used in chemotherapeutic protocols designed to attack cells on the basis of cell kinetic characteristics as G₂-sensitive agents. Used in conjunction and at low doses BLEO and BCNU may improve the synchrony of the neoplastic cells obtained in patients with BLEO alone (3) while increasing the therapeutic index as a result of their different toxic effects.

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REFERENCES

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