Comparison of in Vitro Methods to Determine Drug-induced Cell Lethality

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SUMMARY

In proliferating cell populations, the inability to reproduce indefinitely is the only relevant criterion to assess cell lethality. The in vitro colony formation technique (CF) used to determine reproductive death is, however, too slow and has several technical limitations. For finding suitable, more rapid techniques that assessed drug-induced cell killing, a human lymphoma cell line was exposed in vitro to increasing concentrations of adriamycin, bleomycin, and 1,3-bis(2-chloroethyl)-1-nitrosourea for 1 hr. Survival was assayed immediately after treatment and at regular intervals thereafter. Data from CF were compared to those resulting from the following tests: doubling time, labeling index, dye exclusion, $^{31}$Cr release, and rate of $[3H]$thymidine uptake (scintillation index). Dye exclusion and $^{31}$Cr release failed to demonstrate any killing effect for the 3 drugs. The percentage of killing calculated from doubling time determinations, although dose dependent, failed to correlate with CF. Scintillation and labeling index values displayed similar temporal fluctuations but were not clearly dose dependent and did not correlate with CF. Thus, CF appears as the most reliable, dose-dependent index of cell lethality. Tests that measure metabolic death grossly overestimate or underestimate killing activity induced by 3 of the most effective antitumor drugs.

INTRODUCTION

One approach to individualized antitumor therapy involves screening of potential chemotherapeutic agents to select the drug that will induce maximum cell killing. Various in vitro methods have been used to evaluate cellular damage following exposure to injurious agents: (a) dye exclusion as an indicator of cell membrane integrity (15, 21, 24); (b) chromium release in which radioactive chromate bound to cellular protein is released as a function of cell damage (7, 12); (c) incorporation of radioactive DNA precursors, as an index correlating inhibition of DNA synthesis with cell death (6, 18, 19, 25); and (d) cell proliferation in short-term cultures where loss of exponential growth is associated with cell death (14). However, drug-induced cell lethality may be manifested in 1 of 2 ways: metabolic death and reproductive death. Metabolic death is an event that can be measured by a variety of rather simple techniques that reflect immediate metabolic dysfunction, whereas reproductive death is a delayed process that manifests itself as the loss of unlimited proliferative capacity. In proliferating cell populations, this inability to reproduce indefinitely should be the only relevant criterion to assess cell kill. Since it has been shown that cells rendered incapable of sustaining proliferation may present intact metabolic properties and may complete 1 or several divisions before the entire progeny perishes from the inherited damage (28, 37), methods measuring metabolic death may grossly overestimate or underestimate the viable cell population.

This investigation compared techniques that measure reproductive versus metabolic death in order to determine which, if any, of the latter methods most closely approximate results obtained from assays determining reproductive integrity. In making this comparison, it was hoped that 1 of the more rapidly performed techniques determining metabolic death would emerge as a reliable indirect index of cell viability and thus be more suitable for utilization in routine clinical studies. In the following experiments, the classical in vitro technique determining reproductive death, that of CF (22), was used as a standard index to assess cell lethality after drug exposure.

Three of the most effective antitumor agents, ADR, BLEO, and BCNU, were utilized. ADR is an antitumor antibiotic of the anthracycline group isolated from Streptomyces peucetius var. caesius (1). It is considered that ADR intercalates with DNA (6), thus inhibiting not only DNA-dependent RNA synthesis (33) but also DNA duplication by affecting template-related DNA polymerase activity (32). BLEO is a complex glycopeptide, produced by a strain of Streptomyces verticillus (31). The exact mechanism by which BLEO exerts its lethal effects on cells is still unknown. However, it has been shown to inhibit the synthesis of RNA markedly, to inhibit the synthesis of protein and RNA to a lesser degree (26), and to cause cell progression delay in $G_2$ (3, 5, 29). BCNU is considered to be an alkylating agent (36) and has been shown to inhibit both DNA and RNA synthesis (35). Cell samples were processed during the time of drug exposure to determine simultaneous effects. Two- and 24-hr samples were obtained to observe immediate drug effects. A period of 5 days was arbitrarily selected to monitor expression of delayed damage. This interval was sufficient

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The abbreviations used are: CF, colony formation; ADR, adriamycin; BLEO, bleomycin; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; HBSS, Hanks' balanced salt solution; DT, doubling time; SI, scintillation index; LI, labeling index; $[3H]$TdR, tritiated thymidine.

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for untreated populations to complete at least 2 doubling times and for most cells to undergo at least 3 cell divisions (10).

MATERIALS AND METHODS

Cell Line

Cells utilized in this investigation are from a human immunoglobulin-producing cell line (T1 cells) derived from a patient with lymphocytic lymphoma (30). Cells are maintained as monolayer cultures in Ham's F-10 medium supplemented with 20% fetal calf serum, vitamins, and glutamine. Biological characteristics and harvesting methods have been described previously (9, 11). Under these conditions, the average cell cycle time is 31 hr and the doubling time is 53 ± 2 hr. The pre-DNA synthesis period (G1) is 11 hr, the DNA synthesis period (S) is 10 hr, and the post-DNA synthesis period (G2) is 10 hr.

Drugs

All drugs were obtained from the Division of Cancer Treatment, NIH, National Cancer Institute. ADR (14-hydroxy-daunorubicin) (NSC 123127) was manufactured by Farmitalia Research Laboratories (Milan, Italy), BCNU (NSC 409962) by Ben Venue Laboratories (Bedford, Ohio), and BLEO (NSC 125006) by Bristol Laboratories (Syracuse, N.Y.). All drug solutions were freshly prepared in Ham's F-10 media at the beginning of each experiment (pH 7.0 to 7.2) and were prewarmed to 37 °C before being placed on the cells.

Experimental Protocol to Compare Methods that Assess Drug-induced Cell Lethality

The experimental design is depicted in Chart 1. All experiments were done in duplicate with triplicate samples per dose point. Asynchronous T1 cells were seeded in 60-mm Petri dishes (5 x 10⁶ cells/dish) and were incubated for 48 hr to achieve exponential growth. At that time, cells designated for chromium release experiments were prelabeled with ⁵¹Cr (15 μCi/ml; specific activity, 200 mCi/mg) (New England Nuclear, Boston, Mass.) for 45 min at 37°. After incubation, these cells were washed 5 times with HBSS to remove excess isotope. The supernatant medium from all plates was discarded, and increasing concentrations of drug (ADR, 0.1, 0.25, 0.5 μg/ml; BLEO, 10, 50, 100 μg/ml; and BCNU, 10, 50, and 100 μg/ml) were added simultaneously to all plates for 1 hr at 37°. Control plates consisted of cells exposed only to fresh medium. The drug was decanted and the cells were washed twice with HBSS. Fresh medium was added to the dishes and all dishes were reincubated.

Colony Formation. Cells designated for colony formation assays were harvested immediately. Cell suspensions were counted in an electronic particle counter (Coulter Counter Model ZBI; Coulter Electronics, Inc., Hialeah, Fla.) and appropriate aliquots were replated in Petri dishes so that 50 to 100 colonies would appear after 21 days of incubation. Colonies were then rinsed with 0.9% NaCl solution, stained with 0.5% crystal violet, and examined under a stereomicroscope. Colonies of 50 or more cells were considered to originate from viable cells. The percentage of survival was calculated in reference to controls. The plating efficiency of control cultures (the ratio defined by the number of colonies counted divided by the number of cells initially plated) ranged from 20 to 40% in these experiments.

⁵¹Cr Release. Chromium release assays were performed at 1 and 4 hr following drug exposure. At these times, there was minimal spontaneous release of ⁵¹Cr by T1 cells. Radioactivity of cells and supernatant medium was measured with a Baird Atomic Gamma Ray Scintillation Counter (Baird-Atomic, Cambridge, Mass.). The release of ⁵¹Cr by the cells was defined as a ratio calculated from the activity (cpm) of the supernatant, divided by the total activity detected in the supernatant and the cell suspension.

Release (%) =
\[ \frac{\text{cpm of test supernatant}}{\text{cpm of test supernatant and cell suspension}} \times 100 \]

Results were expressed as the percentage of specific release, defined by subtracting control values from corresponding test point values.

Determination of Growth Kinetic Curves (DT) and [³H]ThDr Incorporation (LI and SI). [³H]ThDr (2 μCi/ml; specific activity, 6.7 Ci/m mole) was added to cultures 30 min before harvesting at 1 hr, 2 hr, and daily for 5 days. Cell counts obtained from each dish were used in calculating growth curve slopes by linear regression analysis.

The remainder of the cell suspension was centrifuged and the supernatant was decanted. The cell pellet was resuspended and washed twice in warm 0.9% NaCl solution to remove excess protein. Aliquots of the resultant cell suspension were adjusted to a final concentration of 2 x 10⁵ cells/ml and were treated with an equal volume of cold 10% trichloroacetic acid. The amount of [³H]ThDr incorporated into the acid-insoluble precipitate was determined with a Packard Tri-Carb liquid scintillation counter (Packard Instrument Co., Downers Grove, Ill.) as cpm/cell x 10⁻⁴. Results were expressed as the ratio of [³H]ThDr incorporated by treated cells over that of concurrently processed control cells (SI). The coefficient of variation of values for replicate control cultures was less than 10%.
Cytocentrifuge preparations of the cell suspension were processed for radioautography by the liquid emulsion method (Ilford K-5; Ilford Ltd., Ilford, Essex, England). Radioautographs were exposed for 7 days and developed in D-19 developer (Eastman Kodak, Rochester, N. Y.). The LI was determined by scoring labeled nuclei of 200 cells (labeled cells > 5 grains). Results were expressed as the ratio of the LI of drug-treated cells over that of concurrently processed control cells. The coefficient of variation for replicate samples was less than 5%.

Dye Exclusion. Plates designated for dye exclusion tests were harvested at 1 hr following drug exposure and daily for 5 days. Aliquots of the cell suspension were incubated with both trypan blue (0.4% in HBSS) and eosin Y (0.2% in HBSS) for 10 min at room temperature.

Negative controls were untreated cells while positive controls consisted to T cells frozen in a dry ice-methanol bath and quickly thawed in a 37°C water bath. Cell suspensions were examined under a bright field microscope. Results were expressed as the percentage of stained cells with respect to the total cells counted.

**RESULTS**

**Colony Formation.** Survival of asynchronous T cells as a function of increasing concentrations of ADR, BLEO, and BCNU is presented in Chart 2. The survival curve of ADR-treated cells shows a dose-dependent exponential decrease with a $D_{50}$ of 0.17 μg/ml (1 hr). Only 3% survivors are measured at a concentration of 0.5 μg/ml. BCNU-treated cells exhibit a threshold-type survival curve with a quasi-threshold dose of 14 μg/ml and a $D_{50}$ of 18 μg/ml (1 hr). At a concentration of 100 μg/ml, only 2% survival is observed. The curve representing survival of BLEO-treated cells is biphasic. Of the cell population, 80% is defined by a $D_{50}$ of 15 μg/ml (1 hr) and the remainder is characterized by a $D_{50}$ of 145 μg/ml (1 hr). Survival was reduced to only 9% after exposure to 100 μg/ml.

**Growth Kinetic Determinations.** Cell counts performed immediately after drug treatment and daily for 5 days are graphed in Chart 3. A dose-dependent decline in the slopes representing growth rate of treated cells is noted for all 3 drugs. For quantification of these changes and to obtain an index that would allow possible correlations with cell survival measured by CF, ratios were obtained by dividing the value for the slope of each dose by the slope of the control (Table 1). The lowest dose of ADR, 0.1 μg/ml, resulted in a value of 25% of control. Less than 1% of control was recorded for cells treated with ADR, 0.5 μg/ml. Values for BLEO-treated cells decreased in a dose-dependent manner from 100% of control (10 μg/ml) to a low value of 55% of control (100 μg/ml). Concentrations of BCNU, 10 and 50 μg/ml, resulted in values of 100% of control for treated cells. A concentration of 100 μg/ml resulted in a sharp decline in cell number (16% of control).

SI. SI values varied markedly for all 3 drugs depending on the day of sample processing (Chart 4). One-hr values for all ADR concentrations were above control; cells treated with 0.1 μg/ml reached a maximum value of 150%. The SI decreased by 2 hr, reaching values of 70% of control for all 3 concentrations at 24 hr. SI values for cells treated with 0.5 μg/ml remained at this level throughout the entire observation period. Cells treated with 0.1 μg/ml showed a SI above control (135%) after 48 hr, which declined to 90% of control on Day 5. A similar pattern of SI fluctuations occurred for cells treated with 0.25 μg/ml. The SI of BLEO-treated cells was above control at 1 and 2 hr and then declined, reaching the lowest values at 24 hr for concentrations of both 50 and 100 μg/ml (80% of control). The SI subsequently increased, with peak values noted on Days 2 and 4. By Day 5, all concentrations approached control values. BCNU-treated cells processed after 1 hr demonstrated SI values of 20, 40,
Table 1
Survival of T~ cells following treatment with chemotherapeutic agents (doubling time)

Results are expressed as a ratio (percentage of control) obtained by dividing the value for the slope of each dose by the slope of the control.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration (μg/ml)</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADR</td>
<td>0.1</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>&lt;1</td>
</tr>
<tr>
<td>BLEO</td>
<td>0.1</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>100</td>
</tr>
<tr>
<td>BCNU</td>
<td>0.1</td>
<td>100</td>
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<tr>
<td></td>
<td>0.5</td>
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<td>1.0</td>
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Dye Exclusion. Trypan blue was incorporated by 54% of the positive control cells, while eosin Y was incorporated by 40% of these cells. Less than 5% of the negative control cells incorporated either dye. Drug-treated cells exposed to trypan blue and eosin Y indicated that, at all drug concentrations and at every day tested, cells were 90 to 100% viable.

Comparison of Methods That Assess Drug-induced Cell Lethality. Table 2 is a composite of the data comparing results of each in vitro method under the assumption that test values reflect cell survival. Listed is the lowest value observed, regardless of time of processing. For each assay, the lowest value was chosen in an attempt to correlate maximum drug-induced deviation from control with results from the CF assay. A dose-dependent decline in survival of ADR-treated cells is detected by both the CF and DT assays. A range of 3 to 70% survival is observed for CF, whereas DT assays yield a reduced range of 1 to 25%. Although dose dependent, the declines in percentage of survival detected by the latter method are not proportional to those of CF, i.e., there is a 4- and 5-fold difference in survival between concentrations of 0.5, 0.25, and 0.1 μg/ml with CF, whereas a 2- and 10-fold decline is noted for the DT assay. The SI of cells treated with ADR was insensitive to changes in concentration as indicated by the narrow range in survival (71 to 80% of control). These values showed no dose or time dependency. The LI of T1 cells exposed to ADR declines in a dose-dependent manner; however, this effect is observed only because values were recorded at different time points for each concentration. Survival of cells treated with all concentrations of ADR as determined by 51Cr release and
DISCUSSION

This investigation sought a rapid, reliable technique sensitive to the lethal effects induced by drug treatment. Because evaluation of the degree of cell kill is 1 of the chief problems of individualized chemotherapy, a survey of systems that presumably predict cell death posed a challenging study that offered immediate clinical application. Lethal effects induced by many antitumor agents occur primarily through direct damage to the genetic apparatus. This damage is effected by a variety of mechanisms: DNA strand breaks (27), intercalation (8), cross-linkage (16), and inhibition of DNA replication (17). In addition to these forms of genome disturbance, there are concomitant drug-induced events that are manifested as loss of cell membrane integrity with resultant leakage of vital cellular constituents (2), loss of organelle function (20), or intracellular production and release of proteolytic enzymes or nucleases (2). Many agents with direct activity on the genetic apparatus also inhibit the synthesis of nucleic acids and protein for varying periods of time, frequently in concentrations unrelated to those causing reproductive death. Although any of these concomitant effects could lead to the loss of reproductive integrity, their contribution to the lethal outcome will be negligible at drug concentrations that affect the genetic apparatus. Furthermore, cellular reproductive death is the result of an interplay between the type, extent, and duration of the injury caused by an agent and the efficiency of repairing such damage by the cell or its progeny. Therefore, assays that measure transitory alterations induced by chemotherapeutic drugs are likely to fail in providing an accurate reflection of reproductive death. Thus, the capacity for unlimited proliferation can be assessed only by monitoring the sustained proliferation of surviving cells; this can be adequately accomplished in vitro by the CF technique. Yet this method also has technical limitations such as differences in cell attachment to the culture vessel, the presence of microcolonies, delays in obtaining results, etc.

In our study, direct correlations between CF and other in vitro methods were not expected, for different parameters were being measured; yet the possibility existed that some degree of correlation would allow predictions of cell viability by the use of a relevant correction factor. The extent of cell damage was examined as a function of short-term drug exposure. An incubation time of 1 hr was selected as representative of exposure times in vivo because most drugs are delivered as bolus treatments that results in effective plasma levels of only 30 min to, at most, a few hr. Short exposure times also preclude possible inconsistent results due to chemical instability and loss of biological effectiveness.

Survival determined by each method failed to correlate with that defined by CF. The increase in DT of drug-treated cells was clearly dose related but did not correspond with survival measured by CF. This absence of correlation is
probably a consequence of several factors inherent in the latter assay. There is no distinction in the counting process between viable cells and those cells that subsequently die in culture. This fact tends to overestimate the viable cell count. Conversely, many chemotherapeutic agents, such as BLEO and BCNU, may induce cell progression delay (3–5, 29). This temporary lag in the multiplication rate increases the doubling time of the treated cell population with respect to the control population. This effect produces results that are indistinguishable from those obtained as a consequence of decreased cell populations due to actual cell kill.

Results of SI experiments could not be compared to those obtained from the CF assay due to the absence of a dose-dependent effect for the 3 drugs. A clear dose-dependent effect is required for predicting the efficacy of a chemotherapeutic agent at different concentrations and for making correlations with dose-dependent assays. The temporal fluctuations of SI values observed for all 3 drugs illustrate the potential errors in estimating survival by this method.

Previous reports implying that the number of [3H]Tdr-labeled cells is proportional to the fraction of viable cells (18, 25, 40) are not supported by the results of this investigation. In no instance did SI values correlate with survival assessed by CF. The SI either overestimated (i.e., ADR, 0.25 and 0.5 μg/ml; BLEO, all 3 concentrations; BCNU, 50 and 100 μg/ml) or underestimated (i.e., ADR, 0.1 μg/ml; BCNU, 10 μg/ml) the proportion of surviving cells. Thus, in spite of the simplicity of the technique, the discrepancies noted with this assay system limit its validity and usefulness in predicting cell survival after drug exposure.

51Cr release experiments were performed to define a sensitive index of cellular damage that would provide a biological measurement amenable to quantitative treatment. 51Cr has been used extensively to label target cells for immunological cytotoxicity assays (13, 38). It has been suggested that 51Cr is covalently bound to basic amino acids of intracellular proteins (23), which leak out of the cell as a consequence of membrane damage. Thus, the extent of target cell damage can be assessed as a function of 51Cr release. It was of interest to observe whether the same principles might be useful in providing estimates of survival of cells exposed to drugs; i.e., treated cells releasing the label in a dose-dependent fashion, above that of the spontaneous release of control cells. Results from 51Cr release experiments with T cells failed to demonstrate dose-time-dependent effects. Furthermore, the degree of release was so modest that this assay appeared completely insensitive in measuring the magnitude of injury caused by chemotherapeutic agents.

Staining of cells with vital dyes has been classically used as a criterion of cell death and as an indicator of cell membrane damage (15, 21, 24). Other investigators have recently indicated the unreliability of such methods to assess cell survival (34, 39). In our study, data generated from dye exclusion experiments on drug-treated T cells resulted in values of 90 to 100% viability for all drug concentrations. This indicates that dye was excluded from reproducibly dead, as well as from living, cells and should caution against indiscriminant use of this technique as a predictive index of cell viability.

This investigation has demonstrated that: (a) assays such as dye exclusion and 51Cr release lack the sensitivity to be used as criteria of cell survival; (b) tests that measure inhibition of [3H]Tdr incorporation such as SI and LI are not necessarily a valid index of cell kill because inhibition may or may not be related to reproductively dead; and (c) assays involving measurements of sho-1-term proliferation are not adequate to distinguish actual cell kill from a temporary lag in the multiplication rate. Therefore, failure to demonstrate correlation with CF implies that in vitro methods determining metabolic dysfunction or short-term growth rate should not be used as criteria to predict loss of unlimited proliferative capacity. Thus, these techniques appear unreliable in selecting the drug of choice for individualized antitumor therapy.

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Comparison of *in Vitro* Methods to Determine Drug-induced Cell Lethality

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