In Vivo Clonogenic Tumor Cell Kinetics following 1,3-Bis(2-chloroethyl)-1-Nitrosourea Brain Tumor Therapy

Mark L. Rosenblum, Kathy D. Knebel, Dolores A. Vasquez, and Charles B. Wilson

The Brain Tumor Research Center, Department of Neurological Surgery, University of California, San Francisco, California 94143

SUMMARY

An in vitro colony formation assay was modified to determine the effects of in vivo 1,3-bis(2-chloroethyl)-1-nitrosourea therapy on tumor cell kill and subsequent clonogenic cell kinetics. The measured surviving fraction must be multiplied by the relative total number of tumor cells for each posttreatment interval in order to eliminate inaccuracies caused by dead cell removal in vivo and the lysis of damaged cells by the disaggregation procedure. The assumptions, limitations, and applications of the technique are discussed.

1,3-Bis(2-chloroethyl)-1-nitrosourea doses of 0.25, 0.50, and 1.00 x dose lethal to 10% of animals resulted in approximately a 1-, 2-, and 3-log cell kill, respectively. Significant proliferation of surviving clonogenic cells was observed after a latency period of approximately 2 days, and the rate of tumor regrowth was dose dependent. The cell-doubling times following treatment with 0.25, 0.50, and 1.00 x doses lethal to 10% of animals were 15, 21, and 38 hr, respectively. The interval to complete repopulation of the clonogenic pool corresponds to the observed increase in animal life span for the 2 larger doses and further validates the assay as a true measure of in vivo chemotherapeutic efficacy.

INTRODUCTION

Information about perturbed tumor cell kinetics and tumor heterogeneity is essential for rational planning of single- and multiple-agent chemotherapy. Methods of analysis should concentrate on the component of the tumor population capable of unlimited proliferation, i.e., the clonogenic cell.

We have developed an in vitro colony formation assay that accurately evaluates the in vivo effects of BCNU on a rat brain tumor model (5, 6). In this paper, the methods of analysis have been further modified to permit the determination of posttreatment clonogenic cell kinetics. We quantitated the effects of various BCNU doses on subsequent tumor growth, both grossly and at the cellular level, to obtain information on tumor cell kill, the repopulation of surviving cells, and the variability in tumor response and regrowth. To our knowledge, this is the only method available for accurate evaluation of the posttreatment cellular kinetics of a solid tumor. No other cellular assay system can differentiate between clonogenic, doomed, and dead cells or adjust for dead cell removal. The assumptions, limitations, and applications of our technique will be indicated.

MATERIALS AND METHODS

Animal Model and General Assay Procedure. An N-methylnitrosourea-induced rat brain tumor was transplanted into the brains of 150-200-g adult male Fischer 344 rats, as previously reported (2). The original tumor was described as a malignant astrocytoma (3). However, this tumor (9L) is now classified as a gliosarcoma because of characteristics acquired during animal and cell culture passage (2). A readily extirpable solid tumor is first evident about 1 week after transplantation. Subsequent tumor growth parameters include a tumor weight doubling time of 41 hr, a cell cycle time of 19 hr, a growth fraction of 0.47, and a cell loss factor of 0.12 for tumors of 2- to 100-mg size (T. Hoshino, personal communication). Untreated animal deaths usually occur 3 weeks after transplantation, when tumors attain a weight of approximately 300 mg.

The complete assay procedure has been described (5). In brief, after approximately 2 weeks, when the tumors are well established, animals are treated with a chemotherapeutic agent. Each experiment also includes untreated tumor-bearing controls. After treatment the tumors are removed and weighed. Specimens are disaggregated for 10 min at 37° with 0.5% trypsin into a single-cell suspension. Various cell dilutions are aliquoted to 60-mm Petri dishes and incubated at 37° with 5% CO₂ in an enriched medium that has been optimized for the in vitro growth of this tumor line (5). During the next 14 days a single cell that has retained the ability to divide will form a colony composed of at least 25 cells. Colonies usually contain 100 to 1,000 cells, although groups of 10,000 occasionally are noted. A comparison of the CFE between untreated tumors and tumors treated with chemotherapeutic agents 30 min to 20 days previously permits a measurement of the surviving fraction of tumor cells according to the following formula:

\[
\text{Measured surviving fraction} = \frac{\text{No. of colonies formed (treated)}}{\text{No. of cells plated (treated)}} + \frac{\text{No. of colonies formed (untreated)}}{\text{No. of cells plated (untreated)}} \]

1 This work was supported by NIH Grant CA-13525, The National Phi Beta Psi Sorority, The Joe Gheen Medical Foundation, and the Association for Brain Tumor Research.

2 Recipient of American Cancer Society Clinical Fellowship CF-3537. To whom requests for reprints should be addressed.

The abbreviations used are: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CFE, colony-forming efficiency; LD₅₀, dose lethal to 10% of animals.
Adjustments to the Measured Surviving Fraction. If we assume that the ratio of treated to untreated colony-forming fractions (Equation A) is similar in vivo, the number of colonies formed is proportional to the total number of clonogenic cells, and the number of cells plated is proportional to the total number of tumor cells. Equation A may be rewritten as:

\[
\text{Measured surviving fraction} = \frac{\text{Total no. of clonogenic cells (treated)}}{\text{Total no. of tumor cells (treated)}} - \frac{\text{total no. of clonogenic cells (untreated)}}{\text{total no. of tumor cells (untreated)}}
\]

By reordering Equation B, we obtain:

\[
\text{Measured surviving fraction} = \frac{\text{Total no. of clonogenic cells (treated)}}{\text{Total no. of clonogenic cells (untreated)}} \times \frac{\text{total no. of tumor cells (treated)}}{\text{total no. of tumor cells (untreated)}}
\]

It is apparent that the measured surviving fraction represents the clonogenic population only if there is a similar number of cells in both treated and untreated tumors. However, the total number of cells in a tumor after treatment will change as dead cells are removed and surviving cells proliferate. Dead cells are defined as cells rendered incapable of "unlimited" proliferation (unable to form an in vitro colony; nonclonogenic) by a cytotoxic treatment. This category includes cells unable to divide successfully, subsequent to therapy, and cells that may multiply a limited number of times (usually less than 5) before ceasing proliferative activity (elsewhere termed "doomed" cells). The removal of such dead cells will in itself increase the measured surviving fraction even in the absence of any cell proliferation. A similar problem occurs if enzymatic procedures lyse damaged cells that were rendered fragile by drug treatment. For elimination of these difficulties, the measured surviving fraction is multiplied by the relative total cell number (the inverse of the right ratio in Equation C) and results in a true measure of the surviving fraction of clonogenic cells at any time after therapy:

\[
\text{Surviving fraction of clonogenic cells} = \frac{\text{Measured surviving fraction} \times \text{total no. of tumor cells (treated)}}{\text{total no. of tumor cells (untreated)}}
\]

The total number of tumor cells is the product of the tumor cell yield and the total weight of the tumor, where cell yield is defined as the number of single cells obtained from the dispersion technique per mg of tumor tissue. This measure of cell density represents the number of cells sufficiently intact to withstand the trypsinization procedure. Therefore, the formula for the relative total cell number may be rewritten as:

\[
\text{Total no. of tumor cells (treated)} = \frac{\text{Cell yield (treated)} \times \text{tumor weight (treated)}}{\text{Cell yield (untreated)} \times \text{tumor weight (untreated)}}
\]

and the proliferation kinetics of clonogenic cells at any time after treatment may be determined as follows:

\[
\text{Surviving fraction of clonogenic cells} = \frac{\text{Measured surviving fraction} \times \text{relative cell yield} \times \text{relative tumor weight}}{(E)}
\]

For clear demonstration of the use of these calculations and the elimination of difficulties imposed by posttreatment dead cell removal or lysis, hypothetical examples are presented in Tables 1 and 2. In both cases the untreated tumor cell yield and the total weight of the untreated tumors were used as a basis for comparison. The untreated cell yield was assumed to be 14, and the total weight of the untreated tumors was assumed to be 16.

Table 1

| Hypothetical example of adjustment for the removal of dead cells following a treatment killing 50% of clonogenic cells, without proliferation of survivors, for a tumor with an untreated CFE of 25%.

<table>
<thead>
<tr>
<th>Effects of treatment</th>
<th>Untreated tumor cells</th>
<th>Immediate</th>
<th>After dead cell removal</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 clonogenic</td>
<td>2 clonogenic</td>
<td>2 clonogenic</td>
<td></td>
</tr>
<tr>
<td>2 killed</td>
<td>12 nonclonogenic</td>
<td>12 nonclonogenic</td>
<td></td>
</tr>
</tbody>
</table>

A. Measured surviving fraction = $\frac{\text{CFE (treated)}}{\text{CFE (untreated)}} = \frac{2/14}{4/16} = \frac{2}{4} \times \frac{16}{14}$

B. Relative total cell number = $\frac{\text{Cell yield (treated)}}{\text{Cell yield (untreated)}} = \frac{14}{16}$

Surviving fraction of clonogenic cells = \[(A) \times (B) = \left(\frac{2}{4} \times \frac{16}{14}\right) \times \left(\frac{14}{16}\right) = \frac{2}{4} = 0.5\]

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Table 2

Correction of measured surviving fraction for lysis of fragile cells

Hypothetical example of adjustment for the lysing of damaged or dead cells by the disaggregation procedure following a treatment killing 50% of clonogenic cells, without proliferation of survivors, for a tumor with an untreated CFE of 25%.

<table>
<thead>
<tr>
<th>Untreated tumor cells</th>
<th>Effect of treatment</th>
<th>Cells persisting after disaggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 clonogenic</td>
<td>2 clonogenic, nonfragile</td>
<td>2 clonogenic</td>
</tr>
<tr>
<td></td>
<td>1 killed, nonfragile</td>
<td>1 killed</td>
</tr>
<tr>
<td></td>
<td>1 killed, fragile</td>
<td></td>
</tr>
<tr>
<td>12 nonclonogenic</td>
<td>6 nonclonogenic, nonfragile</td>
<td>6 nonclonogenic</td>
</tr>
<tr>
<td></td>
<td>6 nonclonogenic, fragile</td>
<td></td>
</tr>
</tbody>
</table>

A. Measured surviving fraction = CFE (treated) = 2/9 = 2 × 16
   CFE (untreated) = 4/16 = 4 × 9

B. Relative total cell number = Cell yield (treated) = 9
   Cell yield (untreated) = 16

Surviving fraction of clonogenic cells = (A) × (B) = (2/4 × 16) × (9/16) = 2/4 = 0.5

CFE is 25%, and one-half of the clonogenic cells are killed by therapy (i.e., surviving fraction of clonogenic cells = 0.5). For simplification, it is assumed that surviving clonogenic cells have not proliferated and that no change in tumor weight has occurred before analysis. It is apparent that the measured surviving fraction does not represent the surviving fraction of clonogenic cells following the removal of dead cells (Table 1) or the lysis of damaged cells that had originated from either the clonogenic or nonclonogenic cell pools (Table 2). However, multiplication of these measured values by their representative relative total number of cells results in an adjusted surviving fraction that indicates the true effects of therapy on the clonogenic population.

Valid comparison of cell yields for tumors of varying sizes requires equivalent disaggregation procedures. Unfortunately, it is difficult to mince small and large tumors to the same extent. Furthermore, trypsinization of very large tumors may result in decreased cell yields due to cell clumping or enzyme exhaustion. In order to eliminate these inconsistencies, analyses were performed on small (<40 mg) representative tumor specimens in all cases. Comparison of untreated tumor cell yields obtained in this manner was determined in 52 animals for 3 different experimental periods. Finally, 3 separate specimens from individual tumors were analyzed for CFE to validate the use of such small samples.

Whenever possible, the determination of therapeutic efficacy should utilize tumors of similar size. This would eliminate possible size-dependent differences in tumor cell kinetics that might alter treatment results. Unfortunately, to date, there is no method available for accurate assessment of the size of murine tumors growing intracerebrally. Furthermore, marked differences in tumor weight exist for animals sacrificed after identical posttransplant intervals (M. L. Rosenblum and T. Hoshino, unpublished observations). Therefore, in order to determine the influence of tumor size on CFE, 24 untreated animals in 2 experiments were evaluated, and the CFE’s for small and large tumors were compared. In these same experiments 27 animals were treated with an LD<sub>10</sub> of BCNU and analyzed 24 hr later. The effects of treatment were compared for small and large tumors.

BCNU Treatment. BCNU was obtained from the Drug Development Branch of the National Cancer Institute, NIH, Bethesda, Md., reconstituted in a solution containing 10% ethanol and 0.9% NaCl, and immediately injected i.p. All animals were treated once with a fraction of the approximate LD<sub>10</sub> (13.3 mg/kg). The measured surviving fractions, tumor weights, and cell yields were determined, and the surviving fraction, relative cell yield, and relative tumor weight were obtained by visual approximation using the mean values and error limits for each posttreatment interval. The relative total cell number for each interval was derived as the product of values obtained from the relative cell yield and relative tumor weight curves. The exponential component of the adjusted curves for the surviving fraction of clonogenic cells was determined by least-squares regression analysis, and the clonogenic cell-doubling time was calculated from its slope.

To evaluate the clonogenic population shortly after therapy with an LD<sub>10</sub> of BCNU, 28 animals in 4 experiments were treated, and their tumors were removed and analyzed 0.5, 1.5, 6, 12, or 24 hr later. For the determination of long-term effects, an additional 43 animals in 3 experiments were followed for 2, 3, 4, 8, 11, 14, 18, or 20 days before sacrifice.

The effects of the 0.50 × LD<sub>10</sub> of BCNU were determined for 37 tumors in 3 experiments. Animals were treated and analyzed 6 hr, 1, 2, 3, 4, 6, or 7 days later.

In order to evaluate the short-term effects of a 0.25 × LD<sub>10</sub> of BCNU, 26 animals in 4 experiments were treated, and their tumors were evaluated 1.5, 6, and 24 hr after therapy. For the determination of long-term effects, 33 additional animals in 4 experiments were analyzed at 2, 3, 4, 6, and 7 days posttreatment.

RESULTS

The cell yield is independent of tumor size as well as specimen size for each 9L culture when analyses are per-
Untreated tumor specimens (<40 mg) were disaggregated, and the cell yields (no. of cells/mg) were compared for "small" and "large" specimens. The influence of total tumor size was determined by comparing the cell yields from specimens obtained from small and large tumors.

<table>
<thead>
<tr>
<th>Tumor culture</th>
<th>Dates</th>
<th>Median specimen wt (mg)</th>
<th>Specimens evaluated</th>
<th>No.</th>
<th>Wt range (mg)</th>
<th>Cell yield&lt;sup&gt;b&lt;/sup&gt; (cells x 10&lt;sup&gt;4&lt;/sup&gt;/mg)</th>
<th>Median tumor wt (mg)</th>
<th>Tumors evaluated</th>
<th>No.</th>
<th>Wt range (mg)</th>
<th>Cell yield&lt;sup&gt;b&lt;/sup&gt; (cells x 10&lt;sup&gt;4&lt;/sup&gt;/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>2/5/75-3/26/75</td>
<td>14</td>
<td>9</td>
<td>6-12</td>
<td>47.6 ± 14.2</td>
<td></td>
<td>12</td>
<td>14-33</td>
<td>37.8 ± 14.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>12</td>
<td>37.8 ± 14.6</td>
<td></td>
<td>11</td>
<td>33-341</td>
<td>42.8 ± 17.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>6/10/75-7/11/75</td>
<td>7</td>
<td>8</td>
<td>4-6</td>
<td>33.8 ± 7.3</td>
<td></td>
<td>8</td>
<td>8-20</td>
<td>31.7 ± 11.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8</td>
<td>31.7 ± 11.3</td>
<td></td>
<td>9</td>
<td>19-120</td>
<td>28.8 ± 10.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>8/13/75-9/11/75</td>
<td>5.5</td>
<td>8</td>
<td>3-5</td>
<td>26.1 ± 15.9</td>
<td></td>
<td>8</td>
<td>3-6</td>
<td>23.8 ± 5.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7</td>
<td>25.0 ± 8.8</td>
<td></td>
<td>8</td>
<td>8-161</td>
<td>27.1 ± 16.9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> No significant difference between "small" and "large" specimens or tumors (Student's t test, p > 0.1).
<sup>b</sup> Mean ± S.D.

In Section A, the CFE for small and large untreated tumors was compared in 2 experiments. In Section B, the log cell kill for small and large tumors was compared in 2 experiments 24 hr after i.p. administration of an LD<sub>10</sub> of BCNU.

**Table 3**

Effect of specimen and total tumor size on cell yield

<table>
<thead>
<tr>
<th>Tumor culture</th>
<th>Dates</th>
<th>Median specimen wt (mg)</th>
<th>Specimens evaluated</th>
<th>No.</th>
<th>Wt range (mg)</th>
<th>Cell yield&lt;sup&gt;b&lt;/sup&gt; (cells x 10&lt;sup&gt;4&lt;/sup&gt;/mg)</th>
<th>Median tumor wt (mg)</th>
<th>Tumors evaluated</th>
<th>No.</th>
<th>Wt range (mg)</th>
<th>Cell yield&lt;sup&gt;b&lt;/sup&gt; (cells x 10&lt;sup&gt;4&lt;/sup&gt;/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>2/5/75-3/26/75</td>
<td>14</td>
<td>9</td>
<td>6-12</td>
<td>47.6 ± 14.2</td>
<td></td>
<td>12</td>
<td>14-33</td>
<td>37.8 ± 14.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>12</td>
<td>37.8 ± 14.6</td>
<td></td>
<td>11</td>
<td>33-341</td>
<td>42.8 ± 17.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>6/10/75-7/11/75</td>
<td>7</td>
<td>8</td>
<td>4-6</td>
<td>33.8 ± 7.3</td>
<td></td>
<td>8</td>
<td>8-20</td>
<td>31.7 ± 11.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8</td>
<td>31.7 ± 11.3</td>
<td></td>
<td>9</td>
<td>19-120</td>
<td>28.8 ± 10.5</td>
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</tr>
<tr>
<td>10</td>
<td>8/13/75-9/11/75</td>
<td>5.5</td>
<td>8</td>
<td>3-5</td>
<td>26.1 ± 15.9</td>
<td></td>
<td>8</td>
<td>3-6</td>
<td>23.8 ± 5.7</td>
<td></td>
<td></td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>7</td>
<td>25.0 ± 8.8</td>
<td></td>
<td>8</td>
<td>8-161</td>
<td>27.1 ± 16.9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> 9L tumor cultures are rejuvenated from a frozen cell stock every 3 months to prevent changes induced by long-term tissue culture (6). Cultures are numbered consecutively.

In Vivo Clonogenic Tumor Cell Kinetics after BCNU

**Table 4**

Effect of tumor weight on CFE for untreated tumors and log kill for treated tumors

<table>
<thead>
<tr>
<th>Experiment</th>
<th>No.</th>
<th>Wt range (mg)</th>
<th>Mean ± S.D.</th>
<th>CFE (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. 18</td>
<td>7</td>
<td>3-31</td>
<td>16.2 ± 12.8</td>
<td>20.6 ± 7.5</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>52-191</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>7</td>
<td>4-116</td>
<td>20.2 ± 12.3</td>
<td>25.3 ± 4.9</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>200-424</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Log cell kill = [log (surviving fraction)] + [log (CFE treated ÷ CFE control)]. Each unit represents a 10-fold decrease in surviving fraction.

The CFE for untreated tumors is independent of the total tumor size over a 100-fold range in weights (3 to 424 mg) (Table 4, Section A). Tumors smaller than 3 mg could not be separated accurately from adjacent brain tissue, and animals usually died when tumors attained a weight of 300 mg, although larger masses were occasionally noted. Within an individual experiment the log tumor cell kill 24 hr after an LD<sub>10</sub> of BCNU is independent of total tumor size over a similar weight range (Table 4, Section B). The differences noted between experiments may be attributed to variability in drug administration, pharmacokinetics, and/or biological factors. Consequently, for accurate representation of results of treatment, multiple experiments have been performed.

The maximum cell kill from BCNU is observed 30 mm after i.p. administration of an LD<sub>10</sub> and no change in the mean measured surviving fraction is apparent over the subsequent 4 days (Chart 1). Between Days 4 and 14, the surviving fraction increases, reaching a plateau at a value of approximately 0.1. The standard errors for each tumor, derived from the evaluation of 10 to 15 plates, were usually less than 10% of the values measured and were small compared to differences observed between several tumors treated identically. The most pronounced disparity among tumors is observed on Days 8 and 11, when surviving fractions differ by as much as 1000-fold between tumors analyzed in the same experiment.

After therapy with an LD<sub>10</sub> of BCNU, tumor weight appears to increase slightly for the 1st 3 to 4 days, followed by a decrease to pretreatment levels by the end of the 1st week (Chart 2). Tumor size decreases further during the 2nd week, reaching a nadir of approximately 40% of pretreatment size on Day 14. This time course corresponds precisely with that observed for the removal of dead cells from...
relative total cell number decreases slightly during the 1st 4 days and decreases further during the 2nd week after treatment in a pattern identical to the change in tumor weight. The adjusted surviving fraction for tumors exposed to an \( \text{LD}_{10} \) of BCNU shows that there is a 2- to 4-day lag period before the clonogenic pool initiates significant repopulation (Chart 3). The exponential portion of this curve was determined by least-squares regression analysis to have a correlation coefficient of 0.99 and a slope of 0.44, indicating a cell-doubling time of 37.9 hr. Repopulation of the clonogenic cell pool is complete by 23 days posttherapy.

The maximum cell kill from a 0.5 \( \times \text{LD}_{10} \) of BCNU is observed at 6 hr after drug administration (Chart 3). No change in the measured surviving fraction is apparent in the 1st 2 to 3 days; thereafter, the surviving fraction increases to 0.3 by Day 7. Marked variability in results is noted again, especially from the 2nd to the 4th day. Tumor weight increases parallel to untreated tumor growth for the 1st 3 to 4 days and appears to plateau thereafter (Chart 4). The cell yield decreases after the 4th day to 70% of control values by Days 6 to 7. The increase in relative total cell number closely approximates the change in tumor weight, with the exception of a slight decrease after the 5th day. The adjusted surviving fraction shows a 1- to 2-day lag period before cell repopulation ensues (Chart 7). The exponential portion of this curve was determined by least squares regression analysis to have a correlation coefficient of 0.99 and a slope of 0.80, indicating a cell-doubling time of 20.7 hr. Clonogenic pool repopulation is complete 7 days after therapy.

Treatment with 0.25 \( \times \text{LD}_{10} \) of BCNU results in mean measured surviving fractions of 0.26, 0.14, and 0.27 for tumors analyzed 1.5, 6, and 24 hr after therapy (no significant difference; \( p > 0.05 \), Student's \( t \) test), respectively.
untreated CFE and cell kill must be independent of tumor weight as has been demonstrated in the present study. The measured surviving fraction must be adjusted to give a true evaluation of the clonogenic cell population, since dead cells are removed in vivo and damaged cells are lysed during disaggregation. The adjustment procedure outlined here requires an accurate determination of the total number of cells obtainable from tumors of variable sizes. Since trypsinization of whole tumors may result in inconsistent

Therefore, it may be inferred that the maximum antitumor activity for the 1st day following a 0.25 × LD_{10} of BCNU occurs by 1.5 hr posttreatment. A slightly lower surviving fraction is observed by Day 2, after which the measured values increase to a plateau at approximately 0.3 (Chart 5). Tumor weight increase parallels tumor growth of controls with a possible slight decrease between Days 5 and 7 (Chart 6). Cell yield decreases only after the 5th day to about 70% of control values. The increase in relative total cell number parallels the change in tumor weight, with the exception of a slight decrease after Day 5. The adjusted surviving fraction shows a 2-day lag before significant cell proliferation (Chart 7). Regression analysis of the 3 points on Days 2, 3, and 4 showed a correlation coefficient of 0.99 and a slope of 1.14 indicating a cell doubling time of approximately 14.5 hr. Repopulation of the clonogenic pool is complete by Day 4.

A summary of the results observed for all 3 BCNU doses appears in Table 5. Increased animal life-span was estimated by extrapolation from the relationship between cell kill and animal life-span (6). Assuming a reasonable median untreated animal life-span of 21 days, the anticipated increased life-span agrees closely with the measured repopulation interval for 0.50 and 1.00 × LD_{10} of BCNU.

**DISCUSSION**

The accurate determination of posttreatment clonogenic cell kinetics using a quantitative cellular assay requires a model system that permits near total tumor extinguishment. Analyses should be performed at frequent intervals until treated animals start dying and must be randomized for the treatment group. Experiments should be repeated multiple times because of variability in this system. Therapy should be administered to tumors of identical size or, alternatively,
Summary of posttreatment kinetics of clonogenic tumor cells surviving BCNU comparison with animal survival and BCNU dose.

<table>
<thead>
<tr>
<th>BCNU dose (fraction of LD_{10})</th>
<th>Cell kill ( % )</th>
<th>( \log_{10} )</th>
<th>Proliferation lag (days)</th>
<th>Repopulation interval( ^a ) (days)</th>
<th>Increase in animal life-span( ^b )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>86.0</td>
<td>0.85</td>
<td>2</td>
<td>15</td>
<td>3</td>
</tr>
<tr>
<td>0.50</td>
<td>97.8</td>
<td>1.65</td>
<td>1-2</td>
<td>21</td>
<td>7</td>
</tr>
<tr>
<td>1.00</td>
<td>99.96</td>
<td>3.44</td>
<td>2-4</td>
<td>38</td>
<td>23</td>
</tr>
</tbody>
</table>

\( ^a \) Doubling time of surviving clonogenic cells after proliferation lag.

\( ^b \) Time from treatment until the number of clonogenic cells returns to untreated levels, i.e., surviving fraction = 1.

\( ^c \) Estimated from previously published cell kill versus animal life-span relationship (6) assuming a median untreated animal life-span of 21 days.
trols (Chart 6), and no increase in animal life-span is observed (Table 5). These results demonstrate the basis for the previously demonstrated cell kill threshold that must be exceeded to detect antitumor activity based on animal survival (6).

The cell yield of a tumor is defined as the number of cells surviving the disaggregation procedure per mg of tumor specimen. A dead or doomed cell with membranes sufficiently intact to resist digestion by trypsin will be included in the cell yield, whereas cells with damaged membranes may be destroyed by trypsinization and not counted, despite occupying volume in the tumor prior to disaggregation. This explains the discrepancy often observed between cell yield, which usually decreases during the 1st week after therapy, and dead cell removal, which occurs primarily during the 2nd week (4). Cell yield may also decrease as a result of an increase in tumor extracellular space due to edema or by the development of giant cells. However, neither edema nor giant cells affect either the measured surviving fraction or the relative total number of tumor cells, since the decrease in cell yield is balanced by an equal increase in tumor weight.

The absence of change in the surviving fraction of clonal cells during the 1st day after any BCNU dose suggests that in vivo repair of BCNU-induced potentially lethal damage does not occur. In fact, the initial period of apparent nonproliferation of the clonogenic pool is approximately 2 days for 0.25 to 1.00 × LD_{10}’s. This proliferation lag may be the result of an induced cell cycle delay. The presence of a latency period suggests that sequential administration of a cell-cycle-specific agent before the 2nd posttreatment may be less efficacious than if the agent is given at a longer interval after BCNU therapy.

The repopulation rate for surviving clonogenic cells depends on the dose of BCNU. The cell doubling time of 38 hr following an LD_{10} is clearly greater than after a 0.50 and 0.25 × LD_{10} (21 and 15 hr, respectively). The difference between the 2 latter values may not be significant, considering the few points analyzed and the large biological variation. The reason for a slower repopulation rate after the largest dose is unclear. Surviving cells may reside a greater distance from vascular structures in an environment less able to support active proliferation due to nutrient deficiencies. As a result, the growth fraction of survivors may be lower after the largest BCNU dose. This would imply that sequential cell-cycle-specific therapy may be more effective following a 0.25 or 0.50 × LD_{10} than after an LD_{10}. However, the slower repopulation rate may be the result of primary cellular factors such as "nonlethal damage" or the selection of cells with longer cell cycle times. "Nonlethal damage" implies that a fraction of clonogenic cell progeny dies due to inherited damage (1). Under such circumstances the fraction of potentially clonogenic cells dividing at any time may actually be large despite slow repopulation, and no difference in growth fraction or susceptibility to subsequent cell-cycle-specific therapy would exist between large and small BCNU doses. On the other hand, if this therapy results in the selection of cell populations with different cell cycle times, the schedules required to attain maximal effect from a subsequent cell-cycle-specific agent would vary, since such drugs should be administered for at least 1 cell cycle (7).

The time to complete repopulation of the clonogenic pool (surviving fraction = 1) should, and does, correspond to the increase in animal life-span anticipated from treatment with a 0.50 and 1.00 × LD_{10} of BCNU (Table 5). This observation lends support to the calculated repopulation rates and further validates the colony formation assay as an accurate indicator of in vivo chemotherapeutic effect. Furthermore, the repopulation interval indicates the longest time that may elapse before additional therapy should be administered in order to achieve a stepwise decrease in the clonogenic population and retain a potential for tumor cure.

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Mark L. Rosenblum, Kathy D. Knebel, Dolores A. Vasquez, et al.


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