Response of 9L Rat Brain Tumor Multicellular Spheroids to Single and Fractionated Doses of 1,3-Bis(2-chloroethyl)-1-nitrosourea

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ABSTRACT

This study was designed to examine the relative effect of each of four fractions of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) against 9L rat brain tumor multicellular spheroids and to compare the results of the cell survival and growth delay assays.

Similar levels of cell kill resulted when BCNU was administered either as single fractions of 1.5, 3.0, 4.5, or 6.0 µg/ml for 1 hr or as one to four fractions of 1.5 µg/ml that were administered sequentially for 1 hr each. Survival was increased if the assay was delayed until 24 hr after drug treatment, which indicates that 9L cells in spheroids recover from BCNU-induced potentially lethal damage.

When BCNU was administered in 1.5-µg/ml fractions, plating efficiencies depended markedly on the interval between the fractions. The 12-hr protocol produced an overall higher cell kill. Fractionation schedules of 24 and 36 hr produced less cell kill than did the other schedules. Survival plateaued for the last three treatments with BCNU in the 36-hr schedule.

Cells in S phase at the time of administration of the initial 1.5-µg/ml fraction of BCNU moved into G1- and G2-M phases by 12 hr after treatment. For time periods longer than 12 hr, cells began to appear in the BCNU-resistant S phase. Thus, the movement of cells into the drug-sensitive and -resistant phases after the first fraction correlates well with the corresponding overall cytotoxic effect produced by treatment with the combined BCNU (1.5 µg/ml) fractions.

For a higher concentration (3.0 µg/ml for 1 hr), maximum cell kill was reached within the 12- to 18-hr interval, after which cell kill plateaued. Cells were not found in the S-phase fraction 12 to 36 hr after the first treatment with 3.0 µg/ml; maximum cell kill for the fractionated protocols resulted at these times. Therefore, BCNU, which is classified as a cell cycle-nonspecific drug, can induce a partial synchrony in 9L spheroid cells, which determines the overall cytotoxicity produced by fractionated BCNU protocols.

Although spheroids did not shrink during or after exposure to BCNU, growth was retarded by treatment with all doses and schedules. An optimum time point for growth delay measurement could not be determined from the data. However, correlations between cell survival and growth delay were obtained with arbitrarily chosen end point volumes of four and ten times the volume at the time of treatment.

INTRODUCTION

The effects of drugs and/or radiation on tumor cells have been studied extensively in vivo and in vitro (6). Even though drugs are administered in fractionated doses in the clinical setting, most experimental studies have examined the effects of single-fraction treatment schedules. Exponentially growing monolayer cultures are not suitable for the study of multifraction protocols; because of confluence, cell proliferation ceases after a few days. Similarly, because environmental conditions contribute to a rather short lifetime in culture, cells in plateau phase are of little value for the study of extended protocols. Thus, most studies of multifraction protocols have been conducted with animal models; the end points of these studies include cell survival, tumor regrowth delay, and cure. However, extrapolation of results from animal studies to a clinical application is difficult because reasons for the success or failure of therapeutic protocols used against animal tumor models generally are not understood.

Spheroids are well suited for the in vitro study of multifractional treatments. Although in some respects the 3-dimensional cellular structure of spheroids makes them more complex than monolayer cells in culture, they are less complex than animal tumor models, which are affected by host responses. Experimental end points for spheroid studies include cell survival and growth delay and, while it is not possible to "cure" a spheroid, complete cell kill in a spheroid is essentially equivalent to cure of a tumor in vivo (4, 12). Thus, results obtained with spheroids can be compared directly to results obtained with both in vitro and in vivo systems.

We report here the results of treating 9L rat brain tumor multicellular spheroids with several concentrations of BCNU* using a 4-fraction protocol. The relative cytotoxic effects of each of the fractions were compared, and the results obtained using the cell survival assay were compared with those obtained using the growth delay assay. Results of a detailed study of the synchrony effects caused by the fractionated treatments are discussed.

MATERIALS AND METHODS

Monolayer Culture. Monolayer 9L cells were grown in CMEM consisting of Eagle’s minimum essential medium supplemented with 10% newborn calf serum, nonessential amino acids, and gentamicin (50 µg/ml). Cells from exponentially growing monolayer cultures were used to initiate all spheroids.

Spheroid Initiation and Culture. Optimization of various factors that affect spheroid initiation and culture has been reported (9). For these experiments, 9L cells were harvested from exponentially growing monolayer cultures. Optimization of factors that affect spheroid initiation and culture has been reported (9). For these experiments, 9L cells were harvested from exponentially growing monolayer cultures. 9L rat brain tumor cells were initiated in the growth medium described above and cultured for 24 hr at 37°C, after which the liquid medium was replaced with fresh growth medium. 4-10 mm diameter spheroids were initiated by pipetting the cells into the drug-sensitive and -resistant phases after the first fraction correlates well with the corresponding overall cytotoxicity produced by treatment with the combined BCNU (1.5 µg/ml) fractions.

The abbreviations used are: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CMEM, complete medium; [3H]dThd, tritiated thymidine; PE, plating efficiency.

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oyer cultures and seeded into 100-mm bacteriological grade Petri dishes containing 15 ml of CMEM. After incubation at 37° for 4 days in a humidified 5% CO₂-95% air atmosphere, spheroids were transferred to spinner flasks and incubated for 3 to 5 more days until they were 250 to 350 μm in diameter, after which they were size sorted by passage through a series of sterile nylon monofilament screens (Tetko, Inc., Elmsford, NY). Spheroids with diameters of approximately 250 μm were used for this study. The cell cycle time for cycling cells in 9L spheroids of this size is approximately 20 hr (approximate duration of phases: G₁, 8 hr; S phase, 8 hr; G₂-M, 4 hr), and the growth fraction is approximately 50% (3).

Disaggregation Technique. Spheroids were rinsed twice with calcium- and magnesium-free Earle’s balanced salt solution, and 10 to 20 spheroids were placed in 5-ml plastic test tubes containing 5 ml of an enzyme cocktail that consisted of 0.02% collagenase II (139 units/mg), 0.05% Pronase (45 proteolytic units-Kaken/ml, B grade), and 0.02% DNase I (7 × 10⁶ dome units/mg, G grade) at 37°. Tubes were shaken gently (about 90 strokes/min) for 30 to 45 min in a Dubonoff metabolic shaking incubator at 37°, after which cell suspensions were filtered and centrifuged, and cells were resuspended in fresh CMEM.

Experimental Protocols. For single-fraction protocols, spheroids were treated for 1 hr with BCNU (1.5, 3.0, 4.5, or 6.0 μg/ml). Immediately after treatment, some spheroids were transferred individually to agarose-coated, 16-mm cluster well dishes. Some spheroids were disaggregated and assayed for survival immediately, while others were disaggregated 24 hr after treatment. For some fractionated treatment schedules, spheroids were exposed for 1 hr to 4 fractions of BCNU (1.5 μg/ml) at intervals of 0, 12, 24, or 36 hr. Growth curve determinations were initiated immediately after each treatment period, while cell survival assays were delayed for 24 hr to allow for possible cellular recovery. For the 2-fraction protocol studies, spheroids were treated with equal concentrations of BCNU (1.5 or 3.0 μg/ml) administered for 1 hr each over periods of 0 to 36 hr; results were compared with the results of treatment with single 1-hr fractions of BCNU (1.5, 3.0, or 6.0 μg/ml).

Cell Survival Assay. Single cells disaggregated from spheroids were counted electronically (Rocoy Instruments, Menlo Park, CA) and seeded into 60-mm Petri dishes prepared 24 hr in advance by adding 5 × 10⁴ heavily irradiated (40 Gy) autologous 9L feeder cells to 4 ml of CMEM. The optimum number of feeder cells had been determined in a preliminary experiment. Dishes were incubated at 37° (5% CO₂-95% air) for 12 to 14 days, after which colonies were fixed, stained with 0.125% crystal violet in methanol, and counted under the microscope. PE was determined from the colony counts, and the surviving fraction was calculated as the PE of treated cells divided by the PE of untreated cells.

Growth Delay Assay. Immediately after treatment, spheroids were rinsed with CMEM and transferred individually to 16-mm wells that contained 0.4 ml of 0.65% agarose overlayed with 2 ml of CMEM; medium was replenished weekly. Periodically during the next 4 weeks, spheroid diameters were measured using a calibrated reticle in the eyepiece of an inverted microscope. Growth delay was defined as the additional time, relative to the growth of controls, required for treated cells to reach arbitrarily chosen diameters of 4 and 10 times their diameter at the time of treatment.

Cytokinetic Analysis. To estimate cell cycle progression after 1-hr treatments with BCNU (either 1.5 or 3.0 μg/ml), spheroids with diameters of 250 to 350 μm were treated for 1 hr with [³H]dThd (0.1 μCi/ml; specific activity, 2.0 Ci/mmol), rinsed twice with calcium- and magnesium-free Earle’s balanced salt solution, and treated for 1 hr with each concentration of BCNU. Immediately after treatment, spheroids were rinsed, refed with CMEM, and then incubated. At various times after treatment, spheroids were disaggregated, and single cells were fixed with 70% ethanol, stained with chromomycin A₃, and sorted on a FACS III flow cytometer (Beckton-Dickinson, Mountain View, CA) (5). The coefficient of variation for the untreated 2C DNA peak was 5 to 7%. Three fractions of approximately 1 × 10⁴ cells each were collected from the left half of the 2C DNA peak, the central S-phase region, and the right half of the 4C DNA peak. Cells from each fraction were digested with 0.5 ml of NCS tissue solubilizer (Amersham, Arlington Heights, IL) for 1 hr at 50°C, after addition of a toluene-based fluor, radioactivity in each fraction was counted using a liquid scintillation counter (Beckman Instruments, Inc., Mountain View, CA).

RESULTS

A similar cell kill was produced by BCNU administered either as single 1-hr fractions of 1.5, 3.0, 4.5, or 6.0 μg/ml or as 1, 2, 3, or 4 sequential fractions of 1.5 μg/ml for 1 hr/fraction with an interval of approximately 5 min (0-hr schedule) between fractions (Chart 1). For all exposures, survival was slightly greater when the survival assay was delayed 24 hr after drug treatment.

When BCNU was administered sequentially in 1.5-μg/ml fractions, cell kill assayed 24 hr after each fraction depended markedly on the interval between fractions (Chart 2). BCNU doses administered 12 hr apart killed more cells than did fractions administered 0 hr apart. In the 12-hr protocol, the second fraction killed cells most effectively. Fractionation schedules of 24 and 36 hr produced less cell kill than did the 0-hr schedule; the last 3 doses of BCNU in the 36-hr schedule produced similar levels of cell kill.

Spheroids treated on the 36-hr protocol grew larger and therefore yielded more cells per spheroid than did spheroids treated on shorter schedules (Chart 3). The number of clonogenic cells per spheroid (Chart 4) was estimated by multiplying the PEs (Chart 2) by the corresponding cell yields (Chart 3). The 0- and 12-hr, 4-fraction protocols and the 12-hr, 3-fraction protocol yielded less than 1 clonogenic cell/spheroid; all other schedules yielded more than 1 clonogenic cell/spheroid, several hundred of which survived after each fraction of the 36-hr schedule.

Because the various protocols required different periods of time to complete, recovery mechanisms and/or cellular proliferation may have influenced the number of clonogenic cells per

\[ 
\begin{align*}
\text{SURVIVING FRACTION} & \quad \text{NUMBER OF BCNU FRACTIONS} \quad \text{OR} \\
0 \quad 0.5 \quad 1.5 \quad 3.0 \quad 4.5 \quad 6.0 & \quad \text{BCNU CONCENTRATION (μg/ml)} \\
\end{align*}
\]

Chart 1. Cell survival after graded single fractions of BCNU (0 to 6 μg/ml) (▲), or after sequential fractions of 1.5 μg/ml (□). All drug exposures were for 1 hr, and cell survival was assayed either immediately (●, ◆) or 24 hr (○, ▼) after treatment. Bars, S.D.
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Chart 2. Plating efficiency 24 hr after 1 to 4 fractions of BCNU (1.5 μg/ml for 1 hr). The 0-hr protocol represents 4 sequential doses given about 5 min apart. In the other protocols, the doses were administered either 12, 24, or 36 hr apart. Bars, S.D.

Chart 3. Cell yield 24 hr after 1 to 4 fractions of BCNU (1.5 μg/ml for 1 hr). Symbols are the same as in Chart 2.

Chart 4. Number of clonogenic cells per spheroid, obtained by multiplying the PEs of Chart 2 by the corresponding cell yields of Chart 3.

Chart 5. Data from Chart 4 redrawn to indicate the timing of the survival assay. The dashed line connects the number of clonogenic cells present at various times after 4 fractions of BCNU given 12 hr apart. The 36-hr point on the dashed line was determined immediately after the fourth fraction.

S-phase cells labeled with [3H]dThd were not present in the S-phase fraction collected 12 hr after treatment with BCNU (1.5 μg/ml) (cells contained essentially no radioactivity; see Chart 8). At all other times, cells were detected in the S-phase fraction. After treatment with BCNU (3.0 μg/ml), the number of labeled spheroid. Therefore, we assayed for clonogenic cells that survived the 12-hr protocol at times comparable to those necessary for completion of the 24- and 36-hr protocols. As a control, spheroids were treated on the 12-hr protocol, and the assay was delayed for a time period equivalent to the 36-hr protocol. Delaying the assay until the time when the 24-hr protocol was complete showed that the yield of clonogenic cells per spheroid was approximately equal for both the 12- and 24-hr protocols (Chart 5, ————). However, control spheroids on the delayed assay schedule had fewer clonogenic cells than did spheroids treated on the 36-hr protocol.

By assaying for survival at shorter intervals, survival during the 4-fraction, 12-hr protocol could be examined more closely; it was confirmed that the second dose killed cells most effectively, while the fourth dose was the least effective (Chart 6). Recovery occurred after each of the 4 doses.

After 2 fractionated treatments with BCNU (1.5 μg/ml), survival decreased for intervals between fractions up to 12 hr and increased thereafter (Chart 7). The 3.0-μg/ml fraction also caused a decrease in cell survival with intervals up to 12 to 18 hr, after which time survival plateaued.

The dashed line connects the number of clonogenic cells present at various times after 4 fractions of BCNU given 12 hr apart. The 36-hr point on the dashed line was determined immediately after the fourth fraction.
measurement could not be determined. Therefore, arbitrary end point volumes of 4 and 10 times the initial spheroid volume were chosen to plot the growth delay for comparison with survival data (Chart 11).

**DISCUSSION**

Each concentration of BCNU in the 4-fraction protocol killed essentially the same number of 9L cells when the fractions were administered sequentially with 0 hr between them. In the 0-hr protocol, the cell kill produced by two 1.5-μg/ml fractions equaled that observed after one 3.0-μg/ml fraction; 3 fractions equal one

cells in S phase also decreased to about 0 by 12 hr and remained at this level for up to 36 hr after treatment (Chart 9).

Although spheroids did not shrink in volume during or after exposure to BCNU, growth was retarded by all doses and schedules (Chart 10). The number of fractions is given outside the parentheses, and the time interval is given inside the parentheses. The single fraction (1.5 μg/ml) of BCNU gave the same curve when the drug was added at 0, 12, 24, or 36 hr after the start of an experiment. Because the growth curves were not parallel, selection of an optimum time point for growth delay...
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Chart 10. Growth curves of 9L spheroids after various treatments with BCNU. Bars, S.D.

Chart 11. Tentative correlations between the number of clonogenic cells per spheroid and growth delay. Only the results of 2 fractions are shown, and arbitrary end-point volumes of 4 and 10 times the volume at treatment were used to determine growth delay. Time between the 2 fractions of BCNU: ○, 36 hr; △, 24 hr; □, 0 hr; ○, 12 hr.

increased cell survival observed in spheroids when the survival assay was delayed 24 hr (Chart 1) appears to be real and has been observed in other experiments. This implies that these cells can recover from potentially lethal damage, proliferate, or undergo some combination of these 2 processes. The existence of some repair process operating in spheroids is also implied by the data in Chart 5 because the rate of increase in clonogenic cells per spheroid after the 12-hr protocol is too great (doubling time, 11 to 12 hr) to be attributed to cell proliferation (doubling time, 18 to 20 hr) unless cells that survive treatment regrow at a faster rate. However, we have no data that indicate that cells grow at a faster rate after BCNU treatment. There were more clonogenic cells found immediately after the fourth fraction of the 36-hr protocol than was found after the fourth fraction of the 12-hr protocol, when an equivalent total recovery period was allowed (Chart 5). Apparently, cells can recover from the damage better, via proliferation and repair, if sufficient time is allowed between doses. Data that relate proliferation rates of surviving cells to accumulated drug concentrations are not available, but the recovery rate of cells after each fraction of BCNU is similar and may actually increase with fraction number (see Chart 6).

Twentyman (10) found that survival of cells from EMT6 spheroids increased after BCNU treatment when the survival assay was delayed 24 hr and that survival levels could be correlated with growth delay times to predict reasonable doubling times for surviving clonogenic cells.

The 12-hr protocol was more effective than was the 0-hr protocol because of the increased cell kill provided by the second dose. When BCNU (1.5 μg/ml) was administered to 9L spheroids as 4 fractions at intervals of 0, 12, 24, and 36 hr, cytotoxicity depended markedly on the interval between the fractions; the 12-hr protocol produced the greatest cytotoxic effect, as measured by both cell survival and growth delay. For 3.0-μg/ml fractions, however, cytotoxicity increased with increasing time intervals up to 12 to 18 hr and then plateaued.

Low concentrations of BCNU (e.g., 1.5 μg/ml for 1 hr) are...
thought to induce minimal perturbations in the rate at which 9L cells traverse the cell cycle, although this has not been shown conclusively. A 3.0-μg/ml dose of BCNU administered for 1 hr does perturb the cell cycle, as judged from flow cytometry profiles (5). 9L cells from exponentially growing cultures exhibit a definite cell cycle age response after treatment with BCNU (2); cells in G₁ and G₂-M phases are more sensitive to BCNU than are cells in S phase. Also, noncycling 9L cells in spheroids are more sensitive to BCNU than are cycling cells (5).

The [³H]dThd labeling study showed that no cells were in S-phase populations analyzed 12 hr after the first fraction of BCNU (1.5 μg/ml) was administered (Chart 8). Therefore, cells surviving the first fraction are predominantly in G₁ and G₂-M phases at 12 hr after treatment and therefore are more sensitive to BCNU. These data confirm that 1.5-μg/ml fractions of BCNU induce minimal perturbations in the cell cycle. The durations of the G₁-M, S, and G₂-M phases in 9L spheroids are approximately 8, 8, and 4 hr, respectively (3). Therefore, unperturbed cells in S phase at the time of treatment with BCNU (1.5 μg/ml) should be in G₁ and G₂-M phases after a 12-hr interval.

After a 1-hr treatment with BCNU (3.0 μg/ml), most cycling cells accumulated in the late S, G₂-M phases between 18 and 48 hr after treatment (5). Therefore, cells in S phase at the time of treatment may move to the BCNU-sensitive G₂-M phase during the 18-hr interval after treatment, where they remain for up to 48 hr. This interpretation agrees with the data reported here that cytotoxicity increases up to 18 hr after treatment and then remains constant (Chart 7). It also agrees with the labeling data (Chart 9), which show that the distribution of cells through the cell cycle remains constant for time periods of approximately 12 to 36 hr after treatment.

The results of an in vivo study can be compared with our results. Rosenblum et al. (7) measured the survival of cells obtained from 9L tumors implanted intracerebrally in rats that were treated with 4 doses of BCNU (one-half of a 10% lethal dose, 6.6 mg/kg) administered 24 hr apart. The first dose reduced cell survival by approximately 2 logs, the second dose reduced cell survival by approximately 1 log, and the last 2 doses produced little additional cell kill. These results are similar to those of the 36-hr protocol reported here and may be explained in part by perturbation of the cell cycle that causes the accumulation of cells in a drug-sensitive phase. Of course, it is also possible that the reduced BCNU activity seen with different fractions in vivo is a consequence of BCNU-resistant tumor cell subpopulations.

The finding that spheroids did not shrink in volume after BCNU treatment, even after high concentrations of BCNU capable of sterilizing spheroids had been administered, suggests that they possess a stable matrix. If approximately 10 clonogenic cells were present, spheroids could repopulate during the observation period (Charts 4 and 10). It is also interesting that similar numbers of clonogenic cells that survive 2, 3, or 4 fractions given 36 hr apart (Chart 4) produced markedly different growth curves (Chart 10). Quantitative assessment of the growth rates of spheroids after various treatments depends on the end-point volume. This phenomenon may be the result of a slow rate of removal of dead cells and debris and/or the result of averaging the growth curves of individual spheroids. Beeg (1) has shown that both processes may be operative in tumors in vivo. Thus, the relationship between cell survival and growth delay is tentative. However, if these relationships hold, it would allow prediction from one assay to the other and might help explain similar results from animal systems.

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