Cancer Chemother Pharmacol (1987) 20: 89–95

Cancer Chemotherapy and Pharmacology

Response to BCNU of spheroids grown from mixtures of drug-sensitive and drug-resistant cells*

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Summary. Multicellular spheroids were grown from mixtures of rat brain tumor cells sensitive (9L) and resistant (R_3) to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). As shown previously, after treatment with 3 μM BCNU, percentages of each cell subpopulation in mixed-cell spheroids were estimated with the sister chromatid exchange (SCE) assay and found to be approximately the same as percentages used to initiate spheroids. The sensitivity of 9L cells in mixed-cell spheroids treated with BCNU, estimated by changes in the number of SCEs induced by treatment, decreased as the percentage of R_3 cells increased. When spheroids were disaggregated into single cells before treatment, however, the number of SCEs induced in the 9L population did not decrease but remained at levels similar to those found for spheroids grown from 9L cells only. These data suggest that the cell-cell interactions that influence BCNU sensitivity in mixed cell spheroids depend on three-dimensional intercellular contact. The response of purely 9L, purely R_3, and mixed-cell spheroids to BCNU was also determined using the cell survival and spheroid growth delay assays. The surviving fractions of individual spheroids treated with 40 μ*M* BCNU were slightly greater than expected; growth delays found for mixed-cell spheroids were 2–3 days less than expected. These findings suggest that cells in mixed-cell spheroids are more resistant to BCNU than would be predicted from the sensitivities of purely 9L and R_3 spheroids.

Introduction

Many animal and human neoplasms consist of discrete cell subpopulations that can be heterogeneous with respect to morphology, DNA content, growth rate, and a number of other biological characteristics [3]. One of the most clinically significant forms of intraneoplastic diversity, however, is the drug sensitivity of the individual cell subpopulations. Recently, it was recognized that this aspect of tumor heterogeneity is a major factor contributing to the failure of the clinical management of cancer [3]. Despite its clinical relevance, the effects of intraneoplastic diversity in drug sensitivity on the treatment response of tumors remains poorly understood, partly because of the lack of adequate models.

In an attempt to develop an in vitro model that simulates a heterogeneous tumor, we have recently begun to study the effect of drug treatment on spheroids grown from mixtures of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)-sensitive (9L) and BCNU-resistant (R_3) rat brain tumor cells [10]. Spheroids approximate many of the characteristics of in situ tumors, including three-dimensional intercellular contact; a range of oxygen tension, pH, and nutrient levels; and the presence of cycling and noncycling cell subpopulations [6]. These characteristics are generally not present in monolayer cell culture systems. In addition, spheroids can be grown and maintained in culture for several weeks under rigidly controlled environmental conditions. By growing spheroids from mixtures of drug-sensitive and drug-resistant cells, one more characteristic of the in situ neoplasm can be added to the spheroid model.

A major technical problem in the design of experimental protocols to examine mixtures of subpopulations with different drug sensitivities has been the inability to determine quantitatively the percentages and responses of cell types within the mixture. In mixed-cell spheroids, the sister chromatid exchange (SCE) assay – a sensitive, relatively simple method for the detection of damage to DNA [13] – can be used to perform these measurements. Induction of SCEs caused by BCNU in 9L monolayer culture cells has been correlated with cell kill; data obtained with the SCE assay correspond to the shoulder region of dose-response cell survival curves [9]. 9L cells resistant to BCNU-induced cell kill are also resistant to the induction of SCEs by BCNU [11]. In addition, agents such as X-rays and *alpha*-difluoromethylornithine that modify cell kill caused by BCNU also modify the BCNU-induced SCEs [7, 8]. Thus, the SCE assay performed after the treatment of 9L cells with BCNU provides the same relative information as the cell survival assay. Results obtained with cell survival assays are based on average values for all the tumor cell subpopulations, and without extensive cloning experiments, it is not possible to use the cell survival assay to differentiate the sensitivities of cell types. The SCE assay, however, is based on the analysis of individual cells and may be applicable to the study of intraneoplastic diversity in drug sensitivity of cell subpopulations. Thus, we conducted experiments in which mixtures of 9L and R_3 cells were treated with BCNU in monolayer culture [12]. Histograms of the number of cells versus SCEs per metaphase clearly showed

* Supported in part by NIH Grant CA-39465-02 and NIH Program Project Grant CA-13525
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the presence of two distinct cell subpopulations in ratios approximately equal to the ratios of cells seeded for the experiments. This use of the SCE assay suggested that it could be used to evaluate the drug sensitivity of cells in spheroids. We grew spheroids from different ratios of 9L and R_3 cells; after treatment with BCNU and disaggregation, the SCE assay was performed. SCE histograms indicated that the percentages of 9L and R_3 cells used to initiate growth of spheroids were maintained in spheroids up to 500 μm in diameter. In mixed-cell spheroids, the number of BCNU-induced SCEs in the sensitive 9L population decreased as the percentage of BCNU-resistant R_3 cells within the spheroids was increased [10]. When experiments were conducted on cells in monolayer culture, however, we found no effect on BCNU-induced SCEs for either cell type [12]. Because changes in SCEs reflect changes in cell drug sensitivity, these results suggested that an interaction between cell subpopulations occurs within spheroids in which the resistant R_3 cells influence the drug response of the sensitive 9L cells. In an attempt to better understand this cell-cell interaction and its significance and to further characterize this spheroid model, we have evaluated the cell survival and growth delay of mixed-cell spheroids treated with BCNU. Results showed that drug sensitivity measured by the cell survival assay was slightly less than predicted by addition of the individual responses of 9L and R_3 cells, and growth delays were 2–3 days less than expected.

Materials and methods

Cells. BCNU-sensitive (9L) and BCNU-resistant (R_3) rat brain tumor cells were grown in monolayer culture in Hsu's medium supplemented with 10% fetal calf serum in a humidified 5% CO_2: 95% air atmosphere at 37° C, pH 7.4. The 9L cell line, originally isolated from a primary brain tumor induced in a Fischer rat by *N*-methylnitrosourea, has been well characterized with respect to its BCNU response [2]. The BCNU-resistant cell line R_3 used in these studies was cloned from the resistant line $9L_2$ and had the same response to BCNU as reported for $9L_2$ cells [10]. Doubling times for R_3 and 9L cells in monolayer culture were each approximately 19 h with modal chromosome numbers of 57 and 55 respectively. The background levels of SCEs per metaphase were approximately 12 for both cell lines. In the original studies, Eagle's minimum essential medium was used. Cells exhibited essentially the same growth and response to drug in Hsu's medium.

Spheroid initiation and culture. Spheroids grown from 9L cells, R_3 cells, or mixed-cell spheroids were initiated by adding single-cell suspensions of cells obtained from exponentially growing monolayer cultures to spinner flasks in specified ratios (3×10^6 cells or mixtures totaling 3×10^6 cells. Culture conditions were the same as those described [2]. After the initial seeding, spinner flasks were incubated at 37° C and rotated at 120 rpm for 5–7 days until spheroids were 100–200 μm in diameter, at which time they were sorted by passage through a series of sterile nylon monofilament screens (Tetko Inc., Elmsford, NY). Spheroids (100–200 μm) were then used for experiments.

BCNU treatment. Spheroids of a specific size were placed into fresh growth medium at a concentration of 5–10 spheroids/ml 24 h before BCNU treatment. BCNU was dissolved in ethanol (95%) and added to spinner cultures maintained at 37°. The amount of vehicle was never more than 0.3% of the treatment volume. After 1 h, the spinner cultures were placed on ice and the spheroids were removed, washed in fresh medium and assayed as described below.

Disaggregation technique. Spheroids were rinsed with calcium- and magnesium-free Hanks' balanced salt solution and placed in 25-ml Erlenmeyer flasks that contained 10 ml enzyme cocktail consisting of 0.02% collagenase II (155 units/mg), 0.05% pronase (91 PUK/mg), and 0.02% DNase I (7.2×10^4 dornase units/mg). Flasks were incubated at 37° C and rotated at 120 rpm for 30–45 min, after which cell suspensions were filtered and centrifuged and then resuspended in fresh growth medium.

Cell survival assay. Immediately after treatment, spheroids were disaggregated into single cells, counted, and seeded into 60-mm Petri dishes that contained 5×10^4 heavily irradiated (40 Gy) 9L feeder cells. After 12 days incubation, colonies were fixed, stained, and counted. Plating efficiency was determined from colony counts, and surviving fractions of the spheroids were calculated.

Growth delay assay. Immediately after treatment, spheroids 100–200 μm in diameter were rinsed with growth medium and transferred individually to 16-mm wells of Costar multiwell dishes. Each well contained 0.5 ml 0.65% agarose overlayed with 2 ml growth medium. Spheroid diameters were measured every 2 or 3 days by using a calibrated reticle in the eyepiece of an inverted microscope. In our experiments, growth delay was defined as the additional time, relative to the growth of controls, required for treated spheroids to reach 10 times their diameters at the initiation of treatment.

SCE assay. Immediately after a 1-h treatment with 3 μ*M* BCNU, spheroids were disaggregated, 1 to 2×10^6 cells were placed into 75 cm^2 plastic tissue culture flasks and 15 ml growth medium containing 10 μ*M* bromodeoxyuridine (BrdUrd) was added. In one experiment, spheroids were disaggregated, cells were seeded for monolayer culture, and 2 h later were treated for 1 h with 6 μ*M* BCNU. Cells were then rinsed, and growth medium containing 10 μ*M* BrdUrd was added. In both experiments, cells were allowed to replicate for 40 h in the presence of BrdUrd, which allows the incorporation of BrdUrd into two DNA strands of one chromatid and only one strand of its sister chromatid. Three hours before harvesting, cultures were treated with 0.04 μg/ml Colcemid. Mitotic cells were shaken from the flasks and treated with 0.075 KCl. Cells were fixed and washed in freshly prepared methanol:glacial acetic acid (3:1). Sister chromatids were differentially stained using the method of Perry and Wolff [5].

Recovery experiments. To determine specific changes in the proportions of 9L and R_3 cells after treatment with BCNU, mixed-cell spheroids (100–200 μm diameter) were treated with 20 μ*M* BCNU, rinsed with fresh growth medium, and placed back into spinner culture. Growth delays (Table 1) for each spheroid were used as the recovery periods. After recovery, spheroids were treated with 3 μ*M* BCNU and the SCE assay was performed.

Table 1. Cell survival after treatment of mixed cell spheroids with 40 μ*M* BCNU

Spheroid type	Surviving fraction ($\times 10^{-2}$)	
	Observed[a]	Expected[b]
9L	0.13 ± 0.12	–
9L : R_3 90 : 10	7.2 ± 0.9	6.3
9L : R_3 75 : 25	26.0 ± 7.0	15.6
9L : R_3 50 : 50	46.0 ± 7.0	31.1
9L : R_3 25 : 75	56.0 ± 9.0	46.5
R_3	62.0 ± 8.0	–

Spheroids (200–300 μm in diameter) were treated for 1 h with 40 μ*M* BCNU, disaggregated, and assayed for colony-forming efficiency

[a] Values represent the mean ± SE of three independent cell survival experiments

[b] See text for calculation of expected values

Results

We have shown that in treatment of mixed-cell spheroids with BCNU, the number of SCEs induced in the 9L population decreases as the percentage of R_3 cells increases [10]. To evaluate the role of three-dimensional intercellular contact on this change in 9L cell response to BCNU, SCE frequency histograms were generated for spheroids treated with 3 μ*M* BCNU and disaggregated immediately and for monolayer cells treated with 6 μ*M* BCNU 2 h after disaggregation from 100 to 200 μm spheroids. Two hours in monolayer culture was required for cells disaggregated from spheroids to become attached to the tissue culture flasks. SCE frequency histograms for cells disaggregated from spheroids and treated with BCNU predicted the approximate proportions of 9L and R_3 cells used to initiate the spheroids (Fig. 1) and, therefore, provided the same information as obtained from intact spheroids. The mean number of SCEs in the 9L population in each mixed-cell spheroid, however, was approximately the same when cells were disaggregated from spheroids before treatment but, as shown [10], decreased as a function of increasing percentage of R_3 cells in intact mixed-cell spheroids treated with 3 μ*M* BCNU (Fig. 2). It is important to note that spheroids were treated with 3 μ*M* BCNU and monolayer cells were treated with 6 μ*M* BCNU to produce approximately the same level of SCEs. To determine the accuracy of the SCE assay in quantitating the proportions of 9L and R_3 cells in the spheroids, the percent of 9L cells predicted in each mixed-cell spheroid by the SCE assay (using frequency histograms) was plotted versus the percent of 9L cells used to initiate each culture (data not shown). A line was fitted using linear least squares analysis. The correlation coefficient for the data in Fig. 1 is 0.988 and for the SCE data in Table 3, 0.998. These correlation coefficients are similar to those found for the SCE data used to analyze mixed-cell spheroids reported in previous publications [10, 12]. Thus, based on cell proportions used to initiate spheroids, the SCE assay is accurate and reproducible with respect to quantitating the percentages of 9L and R_3 cells in mixed-cell spheroids.

Representative cell survival curves generated after treatment of intact 9L, R_3 and mixed-cell spheroids with graded concentrations of BCNU are shown in Fig. 3. All spheroids were 200–300 μm in diameter at the time of

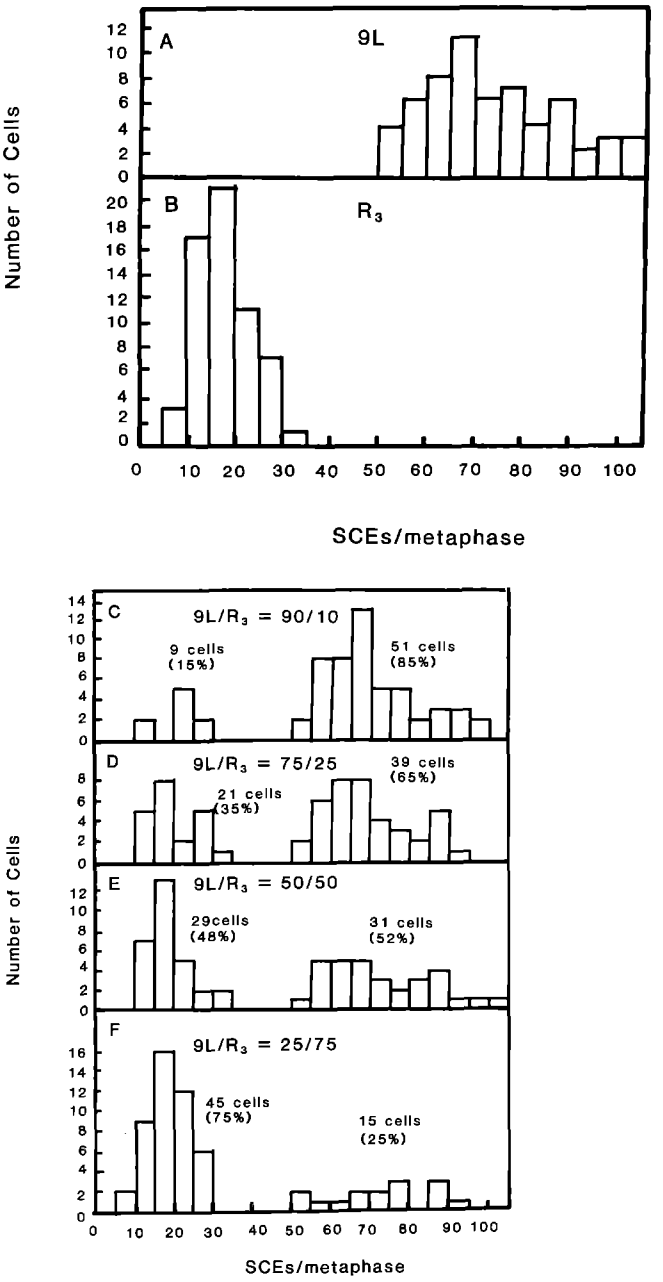

Fig. 1A–F. SCE frequency histograms of the number of cells versus number of SCEs per metaphase for 9L **(A)**, R_3 **(B)**, and mixed-cell **(C–F)** spheroids with diameters of 100–200 μm. Spheroids were disaggregated into single cells; 2 h later they were treated with 6 μ*M* BCNU for 1 h and then assayed for the number of SCEs induced

treatment. No difference in BCNU toxicity is detected for spheroids ranging from 100 to 600 μm in diameter [2]. The plating efficiencies of cells disaggregated from untreated spheroids ranged from 0.42 to 0.48 with no statistical differences between them, and were not dependent on the proportion of either 9L or R_3 cells in the spheroids. As expected, as the percentage of R_3 cells within mixed-cell spheroids increased the surviving fraction after BCNU treatment also increased. For each mixed-cell spheroid, the slope of the curve between 20 μ*M* and 40 μ*M* BCNU was approximately that of the R_3 spheroids. The surviving fractions at 40 μ*M* for mixed-cell spheroids, predicted by ad-

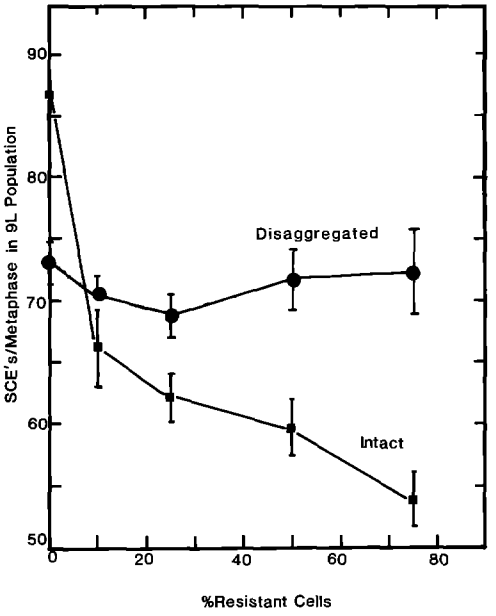

Fig. 2. Plot of the number of SCEs per metaphase in the individual 9L cell populations versus the percentage of R_3 cells. Intact spheroids (100–200 μm in diameter) were treated with 3 μ*M* BCNU, disaggregated into single cells, and assayed for the number of SCEs induced (*squares*). *Circles* represent data obtained for spheroids first disaggregated into single cells, then treated with 6 μ*M* BCNU and assayed for the number of SCEs induced. Values represent the mean ± SE

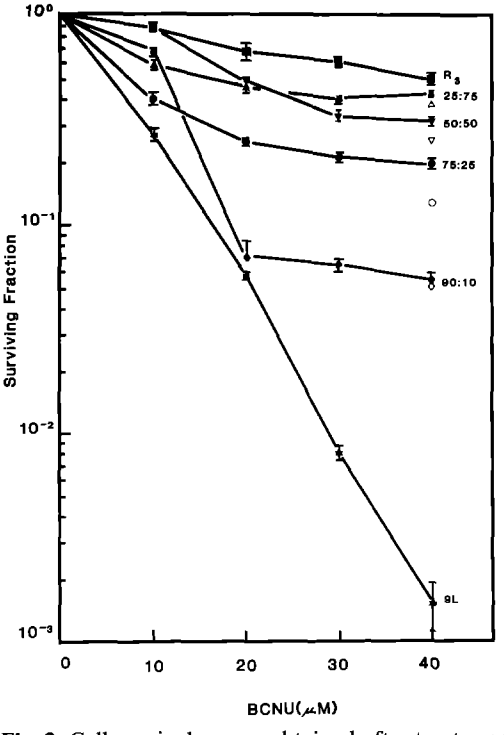

Fig. 3. Cell survival curves obtained after treatment of intact speroids (200–300 μm in diameter) with graded concentrations of BCNU. *Closed symbols* represent the mean of eight Petri dishes; *bars* represent SE. *Open symbols* represent expected survivals for treatment of mixed-cell spheroids with 40 μ*M* BCNU for 1 h when the surviving fractions of 9L and R_3 spheroids are added in the appropriate percentage. *, 9L; ■ R_3; ◆, 90:10 (9L:R_3); ●, 75:25; ▼, 50:50; ▲, 25:75

ding the appropriate percentages of the surviving fractions of purely 9L and R_3 spheroids, are shown by the open symbols in Fig. 3. The surviving fractions obtained in three independent experiments (including those shown in Fig. 3) after 40 μ*M* BCNU treatment of mixed cell spheroids are shown in Table 1. For each spheroid type, the survivals obtained for each mixture at 40 μ*M* are slightly greater than excepted; this suggests that, as predicted by the SCE assay, mixed-cell spheroids are less sensitive to BCNU than predicted based only on the BCNU sensitivities of the individual 9L and R_3 populations.

The effects of treatment of spheroids, like in situ tumors, can be evaluated with a growth delay assay. Results of a typical experiment in which the growth curves of treated and untreated spheroids were compared are shown in Fig. 4. Data were normalized to initial treatment volume. For clarity, error bars have been omitted; the standard errors of the mean were typically between 10% and 20%. Comparison of untreated spheroids shows that 9L spheroids grew faster than R_3 spheroids, and mixed-cell spheroids grew at the rate of 9L spheroids, which suggests that an interaction occurs between 9L and R_3 cells causing equal rates of division [10]. Treatment with 20 μ*M* BCNU caused 25:75 and 50:50 mixtures of 9L:R_3 spheroids to grow at rates similar to those for untreated R_3 spheroids. Growth curves for 9L:R_3 mixed-cell spheroids of 75:25 and 90:10 were biphasic and the slopes of the later portion of curves were similar to the slopes for untreated 9L spheroids. Growth curves for 9L spheroids treated with 20 μ*M* BCNU were also biphasic. The later portion, however, increased at a rate slower than the growth rate of untreated spheroids, which suggests that cells in 9L spheroids do not completely recover from this relatively high BCNU dose.

Growth delays, listed in Table 2, were calculated as the difference in days between treated and untreated spher-

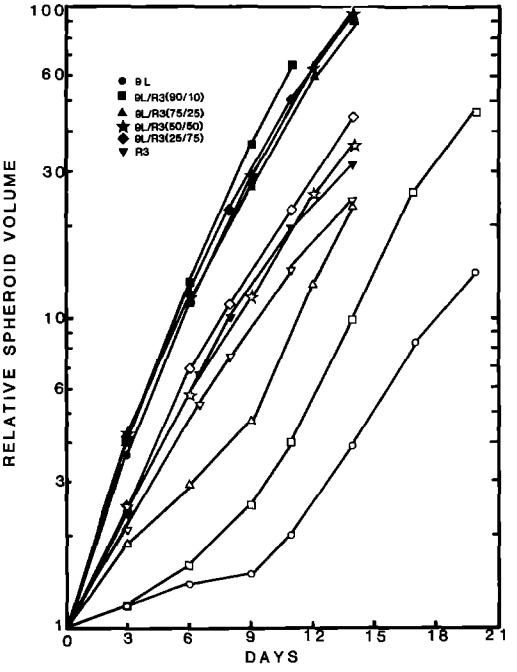

Fig. 4. Growth curves obtained for the various spheroids. Closed symbols represent untreated spheroids and open symbols represent spheroids treated for 1 h with 20 μ*M* BCNU

oids to reach 10 times their initial treated volumes. Values represent the mean of two experiments. Data for one experiment are plotted in Fig. 4. As expected, 9L spheroids had the longest growth delay, and as the percentage of 9L cells in mixed-cell spheroids decreased, the growth delay decreased.

If the growth response of mixed-cell spheroids to treatment with BCNU depends only on the sensitivities of the 9L and R_3 cells, then as a first approximation the growth rate of each treated mixed-cell spheroid can be considered to be the sum of the individual growth rates of the treated 9L and R_3 spheroids. That is, mixed-cell spheroids can be thought of as two spheroids with the volume of each proportional to the percentage of 9L or R_3 cells used to initiate spheroids. Based on this premise, hypothetical growth curves were constructed for treated mixed-cell spheroids by adding the individual volumes of the treated 9L and R_3 spheroids estimated from the proportion of each cell type within the spheroid. For example, the initial mean volume of 50:50 9L:R_3 mixed-cell spheroids was 5.0×10^6 μm^3. Thus, the assumption was made that R_3 cells and 9L cells each account for 2.5×10^6 μm^3. In actual experiments, the initial volumes for treated 9L and R_3 spheroids were 5.2 and 4.7 μm^3 respectively. Because spheroid volume increases logarithmically and is not a linear function, to normalize for the effective treatment volume at zero time, growth curves for purely 9L and R_3 spheroids after BCNU treatment were extrapolated back to initial volumes of 2.5×10^6 μm^3. Beginning at values of 2.5×10^6 μm^3, the volumes of R_3 and 9L spheroids were added at 2-day intervals, converted to relative volume by dividing by 5×10^6 μm^3, and then the theoretical growth curve for 1:1 mixed-cell spheroids treated with 20 μM BCNU was plotted. Growth curves for other mixed-cell spheroids were constructed in this manner. Based on the growth curves of the untreated spheroids (Fig. 4), growth delays at 10 times the initial volume were calculated. For all mixed-cell spheroids, the calculated growth delay was 2–3 days longer than the observed growth delay (Table 2). The similarity between the growth delays obtained in replicate experiments of each spheroid type suggest that the differences between observed and expected growth delays are reproducible and not the result of random errors in the composition of the mixed-cell spheroids. Thus, this variation between observed and expected growth delays suggest that the growth inhibitory effect of BCNU on mixed-cell spheroids is not merely a reflection of the sum of the effects of BCNU on 9L and R_3 cells independently.

Table 2. Growth delay caused by treatment of mixed-cell spheroids with 20 μM BCNU

Spheroid type	Growth delay (days)[a]	
	Observed[b]	Expected[c]
9L	12.4 (12.2, 12.5)	–
9L : R_3 90 : 10	8.7 (8.6, 8.7)	11.2
9L : R_3 75 : 25	5.8 (5.6, 6.0)	7.9
9L : R_3 50 : 50	2.6 (2.6, 2.5)	5.6
9L : R_3 25 : 75	1.9 (1.9, 1.8)	4.2
R_3	1.3 (1.3, 1.2)	–

Spheroids (100–200 μm in diameter) were treated for 1 h with 20 μM BCNU and placed individually into 16-mm wells. The size of the spheroids was then determined every 2–3 days and growth curves constructed

[a] Difference between times taken by treated and untreated spheroids to reach 10 times their own initial volume

[b] Mean of two experiments; values for individual experiments are given in parentheses

[c] See text for calculation of expected values

Table 3. Effect of treatment with 20 μM BCNU on the proportions of 9L and R_3 cells in mixed-cell spheroids

In initial mixtures	Percentages of 9L : R_3 cells	
	Before treatment[a]	After recovery[b]
90 : 10	88 : 12	33 : 67
75 : 25	72 : 28	23 : 77
50 : 50	52 : 48	7 : 93
25 : 75	25 : 75	5 : 95

[a] Spheroids (100–200 μm in diameter) were treated with 3 μM BCNU and assayed for SCEs; the percentages of 9L and R_3 cells were determined from SCE frequency histograms

[b] Spheroids (100–200 μm in diameter) were treated with 20 μM BCNU and allowed to recover for the growth delay periods for each spheroid type listed in Table 2. After recovery, spheroids were treated with 3 μM BCNU and assayed for SCEs; the percentages of 9L and R_3 cells were determined from SCE frequency histograms

Treatment with 20 μM BCNU, which kills approximately two logs of 9L cells, would be excepted to alter the ratio of 9L to R_3 cells in mixed-cell spheroids. Thus, to determine the 9L and R_3 cell percentages in spheroids that have recovered and begun to increase in size after 20 μM BCNU, the recovery experiments described in "Materials and methods", using the growth delays for each spheroid type (Table 2) as a recovery period, were performed. After recovery, the spheroids were treated with 3 μM BCNU and the proportions of 9L and R_3 cells determined from SCE frequency histograms were compared to those determined for a control group of spheroids (not treated with 20 μM BCNU) (Table 3). After treatment with 20 μM BCNU and recovery, there were less than 10% 9L cells in the 25:75 and 50:50 9L:R_3 mixed-cell spheroids. The percentage of 9L cells in the 9L:R_3 mixtures of 75:25 and 90:10 also decreased after recovery, but remained at least 23% in each instance. 9L spheroids that were treated with 20 μM BCNU and allowed to recover for 13 days had slightly fewer SCEs per metaphase after treatment with 3 μM BCNU (70.8 ± 1.5, mean ± SE) than untreated 9L spheroids (86.7 ± 1.9). However, there were no cells in the region of the histogram corresponding to the region for R_3 cells (data not shown).

Discussion

The experimental approach to the study of heterogeneity typically has involved the characterization of isolated tumor cell subpopulations. Though providing important information on the clonal diversity that exists within a tumor and on the basic biology of the individual cell subpopulations, the study of isolated cell subpopulations does not allow consideration of the potential cell-cell interactions within a tumor that may influence both tumor growth properties and drug sensitivity. As Heppner [4] has pointed

out, ideally the drug response of tumor subpopulations should be studied within the context of the tumor and not in an isolated environment.

As part of our investigations of heterogeneity and the effects of cell-cell interactions on tumor chemosensitivity, we have developed an in vitro model in which the SCE assay is used to evaluate the drug response of individual cell types within spheroids grown from mixtures of BCNU-sensitive and -resistant cells [10]. Obviously, because the model uses only two cell subpopulations with different responses to BCNU, it is only an approximation of the intraneoplastic diversity in drug sensitivity that exists within an in situ tumor. Little is known, however, about the effects of heterogeneity on cell sensitivity and the factors that influence it within a tumor. Therefore, results obtained with this simple spheroid model may provide insights that could lead to a better understanding of tumor heterogeneity.

The results of this study show that the presence of R_3 cells caused a decrease in the number of BCNU-induced SCEs in 9L cells when intact mixed-cell spheroids were treated with BCNU, but not when spheroids were disaggregated before treatment. Deen et al. [2] reported that, when measured in terms of cell survival, 9L cells treated as spheroids with BCNU were more sensitive than 9L cells disaggregated from spheroids and treated in either suspension or monolayer culture. In the experiments reported here, in which sensitivity was measured by the number of SCEs induced, treatment of 9L spheroids with 3 μM BCNU for 1 h produced 87 SCEs per metaphase, whereas treatment with 6 μM BCNU after disaggregation of 9L cells from spheroids produced 72 SCEs per metaphase. Thus, with respect to BCNU-induced cell kill and SCEs, 9L spheroids are more sensitive than 9L cells in monolayer culture. The growth fraction of 9L spheroids is considerably less than the growth fraction of 9L monolayer cultures [2]. Recently, it has been shown that noncycling 9L cells, are more sensitive to BCNU than cycling 9L cells [1]. This may explain the difference between the BCNU sensitivities, as measured by SCEs and cell survival, of 9L monolayer and spheroid cultures. However, mixed-cell spheroids grow at the rate of 9L spheroids with no significant shedding of cells occurring for any of the spheroid types (unpublished observation). The growth fractions would not, then, be expected to vary between spheroids, and thus would not appear to be a significant variable in the modification of 9L BCNU sensitivity within spheroids. In addition, we have perviously shown that using the SCE assay the decrease in 9L cell BCNU sensitivity in mixed-cell spheroids occurs in spheroids of 100 μm and 500 μm in diameter [10] suggesting that spheroid size is also not involved in the modification of 9L cell sensitivity. Thus, while it is not possible to eliminate environmental factors completely, the results presented here suggest that the interaction between 9L and R_3 cells depends on the three-dimensional intercellular contact that occurs within a spheroid. The mechanism(s) responsible for this intercellular contact effect remain(s) to be determined.

SCE data suggested that mixed-cell spheroids might be less sensitive to BCNU-induced cell kill than would be predicted on the basis of the individual sensitivities of 9L and R_3 cells. According to BCNU-cell survival curves generated for mixed-cell spheroids, less cell kill actually occurs than would be expected based on the cell killing effects of BCNU on purely 9L or R_3 spheroids. While the observed deviations from expected survival at 40 μM are slight (Fig. 3, Table 1), they occur for each type of mixed-cell spheroid and each deviation from the expected is in the direction of a decrease in BCNU sensitivity. This suggests that the differences between obtained and expected survivals are not due to random errors in the mixing of 9L and R_3 cells during spheroid initiation. In addition, the cell survival assay was performed when all spheroids were 200–300 μm in diameter. Parameters dependent on spheroid size, such as cell cycle distribution, growth fraction, hypoxia, and drug availability, would not then be expected to differentially influence the BCNU response of the various types of spheroids. However, unlike the SCE assay, in which the BCNU sensitivities of the individual 9L and R_3 populations can be individually determined, the cell survival assay is based on the average response of all the cells in the entire treatment population. Thus, although other variables can be discounted, it is not possible to conclusively attribute the increase in observed survival to a cell-cell interaction in which the sensitivity of 9L cells is decreased.

The results reported here show that growth delays as a result of BCNU treatment of mixed-cell spheroids were 2–3 days less than expected. In calculating expected growth delays, we assumed that the growth behavior and BCNU response of the individual populations of 9L and R_3 cells in mixed-cell spheroids were independent of each other. Thus, a cell-cell interaction that affects these values would be expected to produce deviations from the calculated values. With the data available, it is not possible to conclude which, if any, of the 9L/R_3 interactions are responsible for the lowered sensitivity of mixed-cell spheroids to the growth inhibitory effects of BCNU. Results do suggest, however, that the growth interaction, in which 9L cells increased the rate of R_3 division [10], may not be involved. Preliminary results in which the growth rate for spheroids from 9L:R_3 mixtures of 10:90 is similar to the growth rate for R_3 spheroids (unpublished data), suggest that the proportion of 9L cells must be greater than 10% in order to increase the growth rate of R_3 cells. This is in agreement with our results (Table 3, Fig. 4) which showed that treatment of 9L:R_3 mixed-cell spheroids of 50:50 and 25:75 with 20 μM BCNU changed the proportions of cell types such that 9L cells constituted less than 10% of cells within spheroids. After treatment with 20 μM BCNU, these mixed-cell spheroids grew at a rate similar to that of untreated R_3 spheroids (Fig. 4). If the growth interaction between 9L and R_3 cells no longer occurs in 50 9L:50 R_3 and 25 9L:75 R_3 spheroids, then the decrease in the BCNU-induced growth delay may be the result of the R_3 cell-mediated decrease in 9L BCNU sensitivity. However, like the cell survival assay, the growth delay assay does not allow for the analysis of the individual 9L and R_3 sensitivities, but only for the spheroid as a whole. Thus, a change in the BCNU response of a cell subpopulation cannot be specifically evaluated using this assay.

In summary, the data presented here indicate that mixed-cell spheroids are less sensitive to BCNU in terms of cell survival and growth delay than expected. These results are consistent with SCE experiments in which a cell-cell interaction, dependent on three-dimensional intercellular contact, is detected that results in a decrease in the BCNU sensitivity of 9L cells in mixed-cell spheroids. Use

of mixed cell spheroids as an experimental model of a heterogeneous tumor may suggest possible interactions that can occur among tumor cell subpopulations in situ and thus may contribute to the general understanding of tumor response to therapy.

Acknowledgements. We thank Professor Raymond E. Meyn for helpful discussion, Neil Buckley for helpful discussions and for editing the manuscript, and Deborah Thomas Elum for the preparation of this manuscript.

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Received January 30, 1987/Accepted May 22, 1987