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The Effect of Treatment with Single and Split Doses of Spirohydantoin Mustard on the Growth Delay of 9L Rat Brain Tumor Multicellular Spheroids*

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Abstract—*The effects of single and split doses of spirohydantoin mustard on the growth delay and cell survival of 9L rat brain tumor multicellular spheroids have been investigated. Treatment with* 10-25 μM *concentrations of spirohydantoin mustard inhibited the growth of spheroids. Growth delay increased rapidly when assayed during the first* 6-10 days *after treatment, after which a decrease in delay was observed. Plots of the values for the inflection points from growth curves vs drug concentration were linear, and growth delay correlated well with cell survival. There was less growth delay caused by treating spheroids with two* 10 μM *concentrations of spirohydantoin mustard than with a single* 20 μM *concentration. When a* 2 *or* 4 hr *time interval was allowed between treatment with* 10 μM *concentrations of spirohydantoin mustard the delay observed was greater than that obtained with the split dose control. When the resolution of the growth delay assay is considered, the split dose effect can be explained by the existence of a* 5 μM *threshold before any growth delay is observed. Nonetheless, the finding that spheroids treated on the split dose protocol recover from drug damage may have implications for the design of clinical protocols.*

INTRODUCTION

SHM is an antineoplastic agent in which nitrogen mustard is linked through an *N*-ethyl group to a spiroalkyl hydantoin ring system. The drug is a lipophilic alkylating compound [1] that crosses the blood-brain barrier and preferentially concentrates in neoplastic tissue [2, 3]. SHM was designed specifically for the treatment of brain tumors and is currently undergoing clinical trials.

We have studied the cell killing effects of SHM against 9L monolayer cells [4-6] and 9L multicellular spheroids [7, 8]. Results of studies in the spheroid system suggest that SHM induces a G_2/M block in the cycling population [8]. Furthermore, results suggest that non-cycling cells are more resistant in late S and G_2/M phases than in G_1 and/or early S phase [8]. These findings, and the fact that cross-links caused by nitrogen mustard are repairable lesions [9], suggest that splitting the SHM treatment protocol might yield a different response than that caused by treatment with a single dose of the drug. Results of a study designed to examine these possibilities are reported here, with an evaluation of the use of the growth delay assay to determine the effects of split dose protocols on growth of spheroids.

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Abbreviations: SHM, spirohydantoin mustard; FCM, flow cytometry.

MATERIALS AND METHODS

Culture conditions

Culture conditions for the growth of spheroids have been described [7, 10]. Briefly, 3×10^6 9L cells were seeded into glass spinner flasks containing Eagle's minimum essential medium supplemented with 10% newborn calf serum. After an initial incubation period (to allow for spheroid

aggregation and growth) medium was changed three times per week. Spheroids were periodically size-sorted by passage through a series of sterile monofilament screens.

Drug treatment conditions

Spheroids with diameters of 250-400 μm were used. In all experiments spheroids were treated with SHM for 30 min in spinner flasks at 37°C. SHM has a biological half-life of approximately 5 min (unpublished results) and the 30 min treatment assured an infinite exposure to the drug. In split dose experiments spheroids were maintained at 37°C between treatments. For these experiments the split dose control was produced by treating cells for 30 min with 10 μM SHM, rapidly removing the drug-containing medium and rinsing the cells, and treating cells for 30 min with a second, freshly prepared 10 μM concentration of SHM. The interval between treatments was less than 2 min. Concentrations and times used for the various protocols are given in the figures.

Cell survival assay

Spheroids were disaggregated using a published method [10] immediately after the final SHM treatment, and appropriate dilutions of single cells were seeded into 60 mm Petri dishes containing 4 ml of medium and 5×10^4 heavily irradiated (40 Gy) 9L feeder cells. After 12-14 days of incubation at 37°C colonies were fixed, stained with a crystal violet/ethanol solution and counted.

Growth delay assay

Immediately after treatment spheroids were transferred to 16 mm wells of multiwell dishes that contained 0.4 ml of 0.75% agarose and 2 ml of growth medium. Medium was changed weekly and spheroid diameters were measured periodically using a calibrated reticle in the eyepiece of an inverted microscope. At least 12 spheroids were sized for each drug treatment and 24 spheroids were sized for most control determinations. Growth delay was determined from plots of relative spheroid volume vs time as the increased time required for treated spheroids to grow to an arbitrarily chosen size.

RESULTS

Single treatment experiments

Survival and growth response curves for 9L spheroids treated with single doses of SHM are shown in Fig. 1 (A and B). The survival curve (Fig. 1A) had a D_0 of 6 μM and an extrapolation number of 3.5. Treatment with 10-25 μM concentrations of SHM inhibited the growth of spheroids. However, treatment with 5 μM SHM had no effect on growth (Fig. 1B), which suggests the existence of a threshold effect at this drug concentration.

Biphasic curves were obtained when growth delay was plotted vs either time after treatment (Fig. 2A) or relative spheroid volume (Fig. 2B). Both types of curves were characterized by a linear portion with a steep slope (a rapid increase in delay) followed by a linear region, the slope of

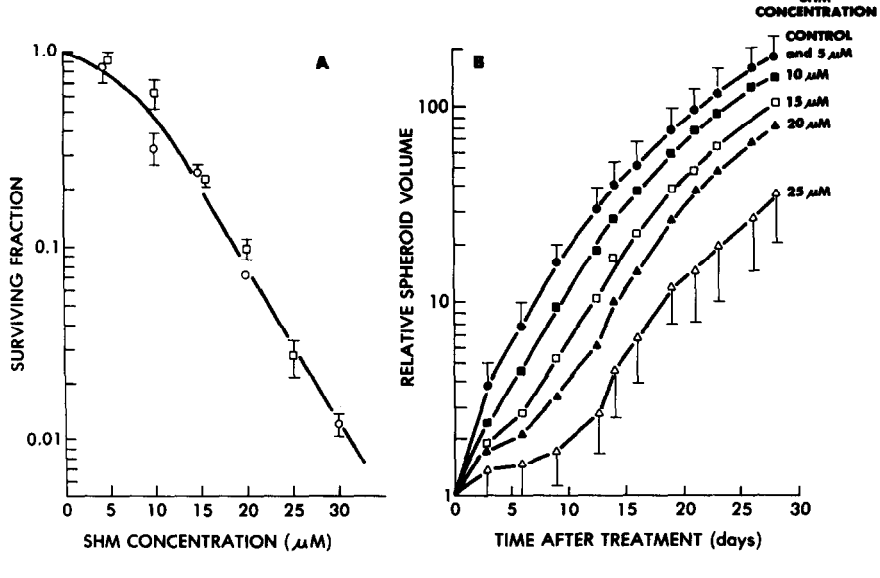

Fig. 1. SHM single dose survival (A) and growth curves (B) for 9L spheroids treated for 30 min with graded concentrations of SHM. The two symbols in the survival plots represent two separate experiments. Error bars represent means ± standard deviations for 4-8 Petri dishes. Each data point for the growth curves represents the relative spheroid volume for 24 spheroids. For clarity, standard deviations are included only for the control and 25 μM data. Error bars for the other data are similar to those shown.

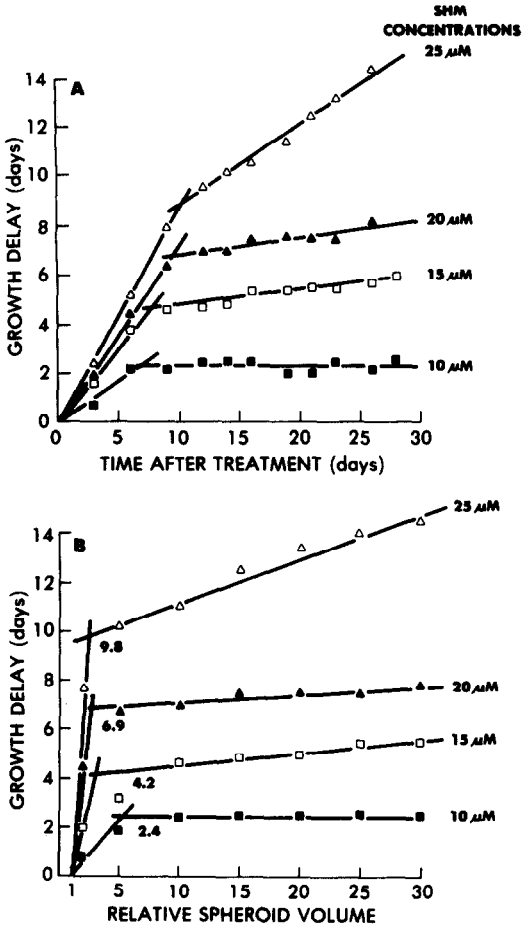

Fig. 2. Growth delay (in days) *caused by SHM plotted vs time after treatment (A) and relative spheroid volume (B). Growth delay data were taken from Fig. 1B; the inflection points on growth delay plots were determined by the intersection of two least squares-fit linear lines through the early and late time points.*

which had a lower value. For these experiments growth delay was taken to be the inflection points on the volume plots such as Fig. 2B. Similar delays could be obtained by taking the inflection points on the time plots such as Fig. 2A or, with the exception of the 25 μM data, by taking the plateau values from either plot. A plot of the inflection points (expressed as days delay; from Fig. 2B) vs SHM concentration was linear for SHM concentrations greater than 5 μM (Fig. 3A). The relationship between growth delay (from Fig. 3A) and surviving fraction (from Fig 1A) is shown in Fig. 3B.

Split dose experiments

The effects on growth delay of treatment with split doses of SHM are shown in Fig. 4. Allowing either 2 or 4 hr between treatments produced growth delays that were greater than the delay produced by the split dose control. For example, at a relative spheroid volume of 5, growth delays for the control, 2 hr and 4 hr data were approximately 3.7, 4.5 and 6 days, respectively. In other experiments an 8 hr interval between treatments produced the same growth delay as a 4 hr interval (data not shown). For reference we have included data for treatment with 10 and 20 μM SHM for 30 min.

DISCUSSION

The results of these experiments show that there is a decrease in cell survival and an increase in growth delay for 9L spheroids treated with increasing single concentrations of SHM (Fig. 1).

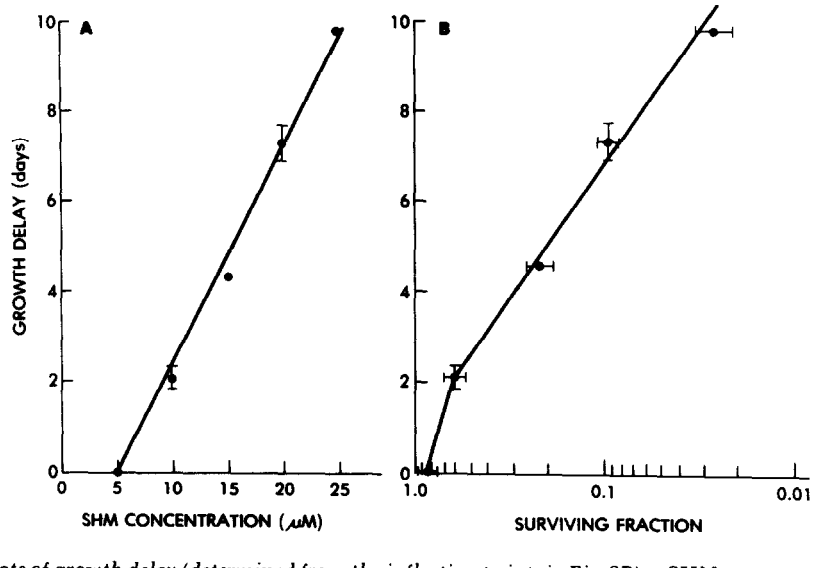

Fig. 3. Plots of growth delay (determined from the inflection points in Fig. 2B) vs SHM concentration (A) and cell survival (B). The vertical error bars in panels A and B represent the standard error for the mean of five experiments (10 and 20 μM only). The horizontal error bars in B represent standard deviations derived from at least four replicate Petri dishes.

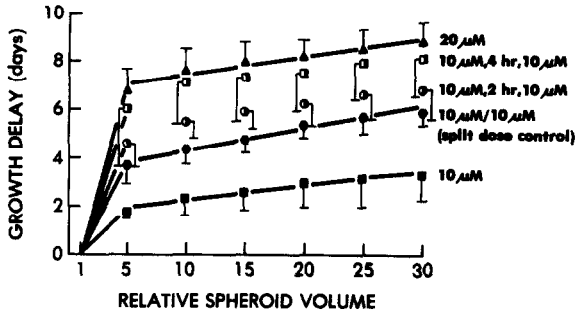

Fig. 4. The effects of split dose SHM treatment on 9L spheroids. The symbols for the 10 and 20 μM data represent the standard error for the mean of five experiments. The data points and standard errors for the 10/10 μM split dose control and the 2 and 4 hr split dose protocols are derived from 2, 4 and 3 experiments respectively. Note that the split dose control (closed hexagons) falls between the 10 and 20 μM single dose treatments.

Growth delay curves are biphasic with respect to either time after treatment or relative spheroid volume after treatment. In an attempt to standardize comparison of treatment protocols, the shift from the rapid increase in delay to a relatively flat portion in the curve at the inflection point was chosen as a representative growth delay for each concentration of SHM. Dose-response curves obtained using this procedure were linear, and a good correlation was found between growth delay and cell survival (Fig. 3). Split dose protocols (10 μM, 2 or 4 hr, 10 μM) caused more growth delay than the split dose control (Fig. 4). The delay, however, was not as great as that caused by treatment with a single 20 μM concentration of SHM.

The correlation between cell survival and growth delay suggests that the early growth delay after SHM treatment is related in part to cell death. However, other factors such as the strong G_2 block caused by SHM treatment [5, 8] might also influence the response of cells during the early phase after drug treatment. The existence of a threshold in both the cell survival and growth delay response suggests that a certain amount of damage must accumulate before an effect caused by SHM is apparent.

All results show that split dose treatment produced less growth delay than did treatment with 20 μM SHM. However, because there is a 5 μM threshold before a linear dose response is evident (Fig. 3A), it would not be unreasonable to expect that the effective concentration in the split dose protocol would be 15 μM. In fact, when the curves for the 15 μM data (Fig. 2A) are compared to the curves for the split dose control (Fig. 4), it is apparent that the growth delay is very similar for both treatment protocols. Therefore, the 'recovery' observed to occur in the split dose control experiments can be explained by the existence of a threshold in the SHM growth delay response. Thus the 2 and 4 hr interval protocols produce more growth delay than the split dose protocol. This apparent increase in damage may be the result of a drug-induced redistribution of cells in the cell cycle that causes a more drug-sensitive population of cells to be exposed to SHM during the 2 and 4 hr treatment protocols.

Because of the inherent variability in the growth delay assay that was found for several experiments, it is not possible to determine with certainty whether the second treatment more effectively produces growth delay than the first treatment. It is apparent from these data, however, that 'true' recovery, relative to the split dose control, does not exist. The fact remains that an effective recovery is obtained when a given total dose of SHM is administered in a split dose protocol, which may have implications for the design of clinical protocols.

REFERENCES

1. Plowman J, Lakings DB, Owens ED, Adamson RH. Initial studies on the penetration of spirohydantoin mustard into the cerebrospinal fluid of dogs. *Pharmacology* 1977, **15**, 359–366.
2. Firemark H, Barlow CF, Roth LJ. The entry, accumulation and binding of diphenylhydantoin-2-^{14}C in brain. Studies on adult, immature and hypercapnic cats. *Int J Neuropharmacol* 1963, **2**, 25–38.
3. Peng GW, Marquez VE, Driscoll JS. Potential central nervous system antitumor agents. Hydantoin derivatives. *J Med Chem* 1975, **18**, 846–849.
4. Bertrand M, Deen DF, Hoshino T, Knebel KD. Recovery from potentially lethal damage induced by spirohydantoin mustard on 9L cells *in vitro*. *Cancer Treat Rep* 1980, **64**, 889–895.
5. Deen DF, Hoshino T, Williams ME, Nomura K, Bartle PM. Response of 9L tumor cells *in vitro* to spirohydantoin mustard. *Cancer Res* 1979, **39**, 4336–4340.
6. Deen DF, Bartle PM, Williams ME. Response of cultured 9L cells to spirohydantoin mustard and X rays. *Int J Radiat Oncol Biol Phys* 1979, **5**, 1663–1667.

7. Deen DF, Hoshino T, Williams ME, Muraoka I, Knebel KD, Barker M. Development of a 9L rat brain tumor cell multicellular spheroid system and its response to 1,3 bis-(2-chloroethyl)-1-nitrosourea and radiation. *JNCI* 1980, **64,** 1373–1382.
8. Sano Y, Hoshino T, Bjerkvig R, Deen DF. The relative resistance of noncycling cells in 9L multicellular spheroids to spirohydantoin mustard. *Eur J Cancer Clin Oncol* 1983, **19,** 1451–1456.
9. Murnane JP, Byfield JE. Irrepairable DNA cross-links and mammalian cell lethality with bifunctional alkylating agents. *Chem Biol Interactions* 1981, **38,** 75–86.
10. Sano Y, Deen DF, Hoshino T. Factors that influence initiation and growth of 9L rat brain gliosarcoma multicellular spheroids. *Cancer Res* 1982, **42,** 1223–1226.