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The Relative Resistance of Non-cycling Cells in 9L Multicellular Spheroids to Spirohydantoin Mustard*

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Abstract—*Surviving fractions of 9L spheroid cells treated with* 1.5, 3.0 *and* 6.0 μg/ml *of spirohydantoin mustard (SHM) decreased when assayed* 6 hr *after treatment but increased thereafter. Flow cytometric analysis showed that exponentially growing 9L monolayer cells treated with SHM accumulated at the* G_2/M *border within* 24 hr. *Cells dissociated from spheroids treated with* 3 *and* 6.0 μg/ml *of SHM accumulated at the* G_2/M *border during the first* 24 hr *after treatment and remained there for the next* 12 hr. *However,* 50% *of the cells remained at the 2C DNA peak. Spheroid cells with 2C DNA content* 24 hr *after treatment were assumed to be non-cycling cells at the time of treatment, and cells that accumulated at the* G_2/M *peak appeared to be cycling cells at the time of treatment; approximately* 50% *of cells in untreated 9L spheroids are in the noncycling pool.* G_1 *and/or early S phase 9L cells in exponential growth elutriated immediately after treatment with SHM had significantly lower* ($P > 0.001$) *plating efficiencies than 9L cells in S and* G_2/M *phases. When spheroids were dissociated, elutriated and plated for colony-forming efficiency* 24 hr *after treatment with* 3 μg/ml *of SHM, fractions enriched in 2C DNA content had significantly higher* ($P > 0.001$) *plating efficiencies than elutriated cells enriched in 4C DNA. These results indicate that SHM is less effective against non-cycling 9L spheroid cells with 2C DNA content than against cycling 9L spheroids cells.*

INTRODUCTION

SHM, 3-(2-[bis(2-chloroethyl)amino]ethyl)-1,3-diazaspiro-[4,5]decane-2,4-dione, is an antitumor compound in which nitrogen mustard is linked through an *N*-ethyl group to a spiro-alkyl hydantoin ring. SHM is a lipophilic alkylating agent [1] that crosses the blood–brain barrier and preferentially concentrates in neoplastic tissue [2, 3].

We have shown that SHM degrades rapidly in serum-containing medium (complete detoxification in 30 min) [4], that the dose–response curves for 9L cells in either exponential or plateau phase have a shoulder followed by a region of logarithmic cell kill [4], that exponentially growing 9L cells accumulated in G_2/M during the 24 hr after treatment with SHM [4] and that recovery from SHM-induced PLD occurs in EBSS or depleted medium within 18 hr after treatment [5].

Because the drug effectively kills monolayer cells and is known to penetrate nervous system tissues, we expected that it would be efficacious *in vivo*. However, SHM did not increase the survival of rats bearing the intracerebral 9L tumor [4].

In this study we used 9L multicellular spheroids to study the effects of SHM on aggregated cells. The cell cycle parameters of 9L

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Abbreviations: SHM, spirohydantoin mustard; CENU, chloroethylnitrosourea; PLD, potentially lethal damage; EBSS, Earle's balanced salt solution; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; MEM, Eagle's minimum essential medium; CMEM, complete growth medium; [$_3$H]-TdR, tritiated thymidine; CFE, colony-forming efficiency; PE, plating efficiency; SF, surviving fraction; DMSO, dimethyl sulfoxide; FCM, flow cytometry.

monolayer and spheroid cells are similar; however, spheroid cells have a fairly constant growth fraction of approximately 0.5, which is similar to the growth fractions of *in situ* 9L brain tumors [6]. We investigated SHM cytotoxicity and DNA perturbations in spheroids, and the cell cycle age response of both spheroid and monolayer cells. Centrifugal elutriation was used to study the differential effects of SHM on cycling and non-cycling cells [7].

MATERIALS AND METHODS

9L monolayer cells and multicell spheroid culture

The 9L monolayer cell and spheroid cell systems and culture conditions have been described [6]. Briefly, 3×10^6 9L cells were seeded into 250 ml of CMEM in glass spinner flasks (Bellco Glass, Vineland, NJ). CMEM consisted of Eagle's MEM supplemented with non-essential amino acids, 10% newborn calf serum, HEPES (25mM) and gentamicin (50 μg/ml). All suspensions were maintained at 37°C and rotated at 180 rev/min by placing the spinner flasks containing a stir bar on a magnetic stirrer inside an incubator (Associated Biomedic System, Inc., Buffalo, NY). At the beginning of week 4, spheroids of 200–400 μm diameter were size-sorted by passage through nylon monofilament screens (Tetko, Inc., Elmsford, NY), transferred to a new spinner flask and used for experiments. The cell cycle time of cycling cells in 9L spheroids under these conditions is approximately 20 hr. The growth fraction, defined as the fraction of cells labeled after 24 hr of continuous exposure to [^{3}H]-TdR, is approximately 0.5 when grown in medium containing fetal calf serum [6], and has the same value if grown in medium containing newborn calf serum.

Disaggregation procedure and CFE assay

Monolayer cells were trypsinized and processed as described [8]. Spheroids in a small volume of CMEM were rinsed twice with calcium- and magnesium-free EBSS. After aspiration of the EBSS, 10–20 ml of an enzyme cocktail {0.05% of pronase [45 PKU (proteolytic units/Kaken)/ml, B grade (Calbiochem Co., La Jolla, CA)], 0.02 of DNase I [7×10^4 dornase units/mg, G grade (Sigma Chemical Co., St. Louis, MO)], 0.02% of collagenase II [139 units/mg (United States Biochemical Co., Cleveland, OH)]} were added to the spheroids in a 25 ml Erlenmeyer flask. The flask was placed in a Dubnoff metabolic shaking incubator at 37°C and shaken gently (approximately 90 strokes/min) for 40 min. The dissociated spheroid suspension was poured through a 100-μm mesh nylon blood filter into 40 ml of CMEM and centrifuged for 5 min at 1000 rev/min in a refrigerated centrifuge. The supernatant was decanted and cells were resuspended in fresh CMEM. The cell suspension was counted electronically (Royco Instruments, Inc., Menlo Park, CA), diluted and seeded into 60-mm plastic Petri dishes containing 4 ml of CMEM and 5×10^4 heavily irradiated feeder cells. After incubation at 37°C in a humidified 5% CO_2–95% air environment for 12–14 days, colonies were stained with a 0.2% ethanolic solution of crystal violet and counted. The PE was calculated as the ratio of the number of colonies counted to the number of cells plated. SF was calculated as the ratio of the PE of treated cells to the PE of untreated cells.

Drug treatment

SHM was dissolved in DMSO, then diluted with fresh CMEM. Monolayer cells were treated as described [8]. Immediately after dilution, 1 ml of various stock SHM solutions was added to spheroids growing in the spinner flasks. The final concentration of DMSO in the drug treatment flask was less than 0.2%, which is non-toxic to 9L cells. Spheroids of 200–400 μm diameter were divided into 4 groups and treated with 0, 1.5, 3.0 and 6.0 μg/ml of SHM and incubated in spinner flasks at 37°C. Because SHM is almost completely detoxified in CMEM within 1 hr, washing of cells or addition of fresh medium was avoided [4]. After 1, 6, 24 and 48 hr, 20 ml of the spheroid suspension were aspirated from each group and spheroids were disaggregated for the CFE assay.

Centrifugal elutriation

Two-day-old monolayer cultures were treated for 30 min with 3 or 5 μg/ml of SHM. After treatment they were injected into the mixing chamber of a Beckman elutriation system (Beckman Instruments, Inc., Palo Alto, CA) [9]. Separation was obtained by holding the flow rate at 32 ml/min and reducing the rotor speed from 2100 to 1600 rev/min in decrements of 50 or 100 rev/min. The elutriation medium was held on ice and the centrifuge was maintained at 2°C. After separation, cells were plated for CFE. In this study approximately 90% of cells were recovered.

Twenty-four hours after treatment with 3.0 μg/ml of SHM, spheroids were harvested, dissociated into single cells, and approximately 8×10^7 single cells suspended in 5 ml of MEM were elutriated in the Beckman system by reducing the rotor speed from 2500 to 1500 rev/min in decrements of 50 or 100 rev/min and then plated for CFE. Approximately 80% of injected cells were recovered in the elutriated fractions. Untreated spheroids were harvested, dissociated, elutriated and plated for CFE and analyzed for DNA content. Flow

cytometry was used to quantitate the purity of the populations elutriated.

Flow cytometry

Disaggregated cells (approximately $1-2 \times 10^6$) were fixed in 70% ethanol for more than 30 min and stained with chromomycin A_3 (Calbiochem, Anaheim, CA) as described [7]. Routinely, 1×10^5 cells were analyzed to obtain a DNA histogram. DNA content was determined on a FACS III flow cytometer (Becton-Dickinson, Mountain View, CA) with a 5-W argon laser set at 457 mm and adjusted to emit 160–200 mW. The resulting fluorescence was passed through a Schott KV-520 short-wave cut-off filter. With these settings, a coefficient of variation of 2.0% for 2.0-μm latex microspheres (Duke Scientific, Palo Alto, CA) and of 6–7% for the G_1 peak of untreated 9L cells were obtained. The computer program developed by Dean [10] was used to calculate fractions of cells in each phase of the cell cycle.

Autoradiography

To confirm the existence of non-cycling cells in the 2C DNA cell population of 9L spheroids 24 hr after treatment with SHM, spheroids were incubated first with 0.05 μCi/ml of [^{3}H]-TdR for 24 hr, a concentration of labeled compound that is not toxic in this system [11], and then treated with 3 μg/ml of SHM. Twenty-four hours after treatment with SHM, when about half the cells had accumulated in G_2/M, spheroids were dissociated, fixed and stained with chromomycin A_3, and sorted by FCM, cells with 2C and 4C DNA contents were collected for the autoradiography study. Cells containing 4 or more grains per nucleus were defined as being labeled, and the labeled populations were determined by scoring 1000–3000 cells from each slide.

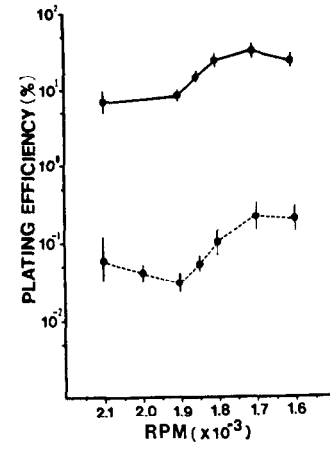

Fig. 1. PE of elutriated monolayer 9L cells treated with 3 (——) or 5 μg/ml (– – –) of SHM. PEs of elutriated untreated cells with 65.3 ± 1.3% (S.E.). (Result of 3 experiments.) Fractions of elutriated cells at each rotor speed are summarized in Table 1. (Results of 4 experiments.)

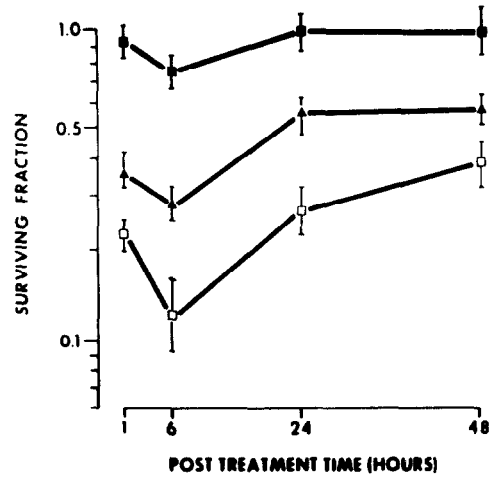

Fig. 2. Time-dependent SF of cells treated as multicullular spheroids with 1.5 (■), 3.0 (▲) and 6.0 (□) μg/ml of SHM. Error bars show standard deviations. (Result of 3 experiments.)

RESULTS

The PEs of elutriated monolayer cells immediately after treatment with 3 or 5 μg/ml of SHM indicate that cells in G_1 (2100 rev/min) and early S phase (1850 rev/min) are more sensitive to SHM than cells in late S and G_2/M phase (1800 rev/min) ($P > 0.01$) (Fig. 1). The percentage of cells recovered in the various elutriated fractions are shown in Table 1. Approximately 80–90% of cells were recovered in each experiment.

The SFs of cells disaggregated from spheroids treated with 1,5, 3.0 and 6.0 μg/ml of SHM were 0.92, 0.36 and 0.25, respectively, at 1 hr and 0.75, 0.28 and 0.12 at 6 hr after treatment. When spheroids were allowed to remain in growth medium for up to 48 hr after treatment with SHM, SFs increased to 1.0 (1.5 μg/ml SHM), 0.57 (3.0 μg/ml SHM) and 0.38 (6.0 μg/ml SHM), while untreated spheroids had PEs of approximately 50% throughout the 48-hr incubation period (Fig. 2).

When spheroids were treated with 1.5 μg/ml of SHM, post-treatment DNA distributions showed a gradual increase in S and G_2 cells and a transient accumulation at the G_2/M border at 24 hr. Cells treated with 3 μg/ml SHM accumulated at the G_2/M border during the first 24 hr and remained there for the next 12 hr (Fig. 3). The ratio of cells remaining at the 2C DNA position to those accumulating at the 4C DNA position was close to unity 24 hr after treatment. The results of the CFE assay for each fraction of cells elutriated 24 hr after treatment with 3 μg/ml of SHM are plotted in Fig. 4. Cells from untreated spheroids had PEs of approximately 50% for the elutriated populations

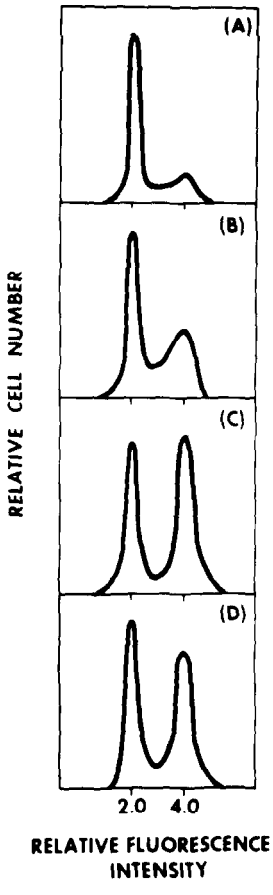

Fig. 3. Representative DNA histograms of 9L spheroids treated with 3.0 μg/ml of SHM: (A) 0 hr (2C = 69%, 4C = 9%); (B) 12 hr (2C = 57%, 4C = 28%); (C) 24 hr (2C = 45%, 4C = 44%); (D) 36 hr (2C = 46%, 4C = 38%).

*Table 1. Percentage of cells recovered in each elutriation fraction**

Fraction (rev/min)	Monolayer Treated with 3 μg/ml SHM‡	Spheroid† Untreated control	Spheroid† Treated with 3 μg/ml SHM§
2500	—	16	2
2400	4	16	3
2300	—	9	5
2200	8	8	8
2100	19	9	7
2000	—	6	7
1900	24	5	10
1850	8	—	—
1800	8	3	11
1700	8	3	11
1600	8	2	10
Washout	7	4	8
Total	94	81	82

*1×10^8 cells injected.
†Average size of cells in 9L spheroids is smaller than cells in monolayer culture. Therefore different elutriation protocols are used.
‡Elutriation performed immediately after a 30-min treatment.
§Elutriation performed 24 hr after SHM treatment. Cells were not rinsed after addition of the drug.

from 2500 rev/min (sedimentation velocity, S_{max} = 9.9 mm/hr. g) [9] through 1600 rev/min (S_{max} = 24.1 mm/hr. g) of rotor speed.

PEs of cells disaggregated and elutriated (2500–1600 rev/min) from spheroids 24 hr after treatment with 3 μg/ml of SHM varied from 45 to 12% (Fig. 4). PEs of the cells elutriated at 1700–1600 rev/min were significantly lower than PEs of the cells elutriated at 2500–2200 rev/min ($P > 0.01$). Cells elutriated at 2500–2200 rev/min were enriched with 2C DNA content, whereas cells elutriated at 1700–1600 rev/min were enriched with 4C DNA content (Fig. 5).

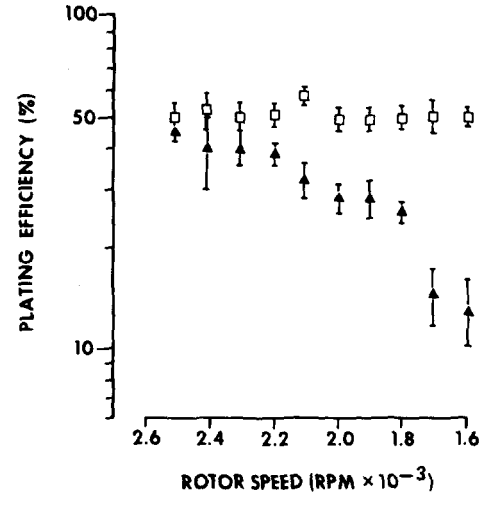

Fig. 4. SF of elutriated cells with (▲) or without (□) SHM treatment at each rotor speed. Fractions of elutriated cells at each rotor speed are summarized in Table 1. Error bars show standard deviations.

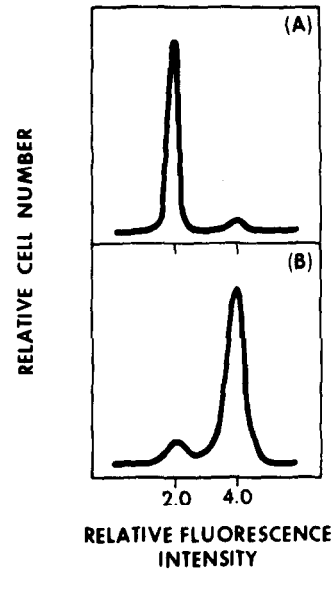

Fig. 5. DNA histogram of elutriated cells at: (A) 2400 rev/min (2C = 91%) *and* (B) 1600 rev/min (4C = 84%). *Coefficients of variation for each peak were:* 7.6% (A), 7.9% (B).

Autoradiographs showed that sorted spheroid cells with either 2C or 4C DNA complements 24 hr after SHM administration had LIs of 29.0 ± 4.6% and 99.8 ± 0.2% respectively (results of two experiments).

DISCUSSION

SHM is a member of the nitrogen mustard class of antitumor alkylating agents, and kills cells in either exponential or plateau phase [4]. A 1-hr treatment with 6–7 μg/ml of SHM killed 2–3 logs of 9L monolayer cells. Therefore we expected to find an increase in life span when rats bearing the 9L tumor were treated with SHM, but no increase in life span was found when rats were treated with various single doses of SHM (1–16 mg/kg). Similarly, when 9L multicellular spheroids were treated with SHM, less cell kill was obtained than with 9L monolayer cells [4].

We assumed that the relative resistance of 9L spheroids and *in situ* tumors to SHM was due to the presence of non-cycling cells that exist in 9L spheroids and *in situ* tumors because 9L cells can repair SHM-induced PLD if they do not initiate DNA synthesis within a reasonably long period (approximately 18 hr) after treatment [5]. When exponentially growing 9L monolayer cells were treated with more than 3.0 μg/ml of SHM, cells tended to accumulate in mid-S and then move synchronously to G_2/M, where the majority of cells were blocked at the G_2/M border 24 hr after treatment [4]. A similar accumulation in the G_2 phase was observed when 9L spheroids were treated with 1.5–6.0 μg/ml of SHM; however, not all cells appeared to be cycling: about half remained in the 2C DNA peak 24 hr after treatment. Accumulation was not complete in spheroids treated with 1.5 μg/ml of SHM, and was delayed in spheroids treated with 3.0 μg/ml or more of SHM. Thus a major difference between the perturbation patterns of 9L spheroids and 9L monolayer cells is that a considerable number of cells in spheroids still remained at the 2C DNA peak even 24 hr after treatment. We assumed these cells were in the non-cycling pool at the time of treatment because cycling cells should accumulate at the 4C DNA peak within 24 hr [4] and because SHM-treated 9L cells in either depleted medium or MEM did not accumulate at the G_2/M border [5]. Most of the non-cycling cells in spheroids, and possibly a few cells in G_1 that did not initiate DNA synthesis, could repair their PLD within 24 hr after treatment and become capable of forming colonies. However, the G_1 phase cells in monolayer culture were most sensitive to SHM and should be arrested in the G_2/M phase.

Autoradiographs showed that at least 70% of cells that remained in the 2C DNA peak after treatment with SHM did not cycle for 48 hr. Labeled cells in this peak may include some cycling cells that were not affected during drug treatment and cells that were cycling during treatment with [^{3}H]-TdR but were not cycling during drug treatment, because cells obviously move from the cycling to the non-cycling pool. Unfortunately we cannot measure how many of the labeled cells in this population were already in the non-cycling pool at the time of SHM treatment. However, at least 70% of cells in the 2C DNA population should be non-cycling, because 100% of cells in the 4C DNA population were in the cycling pool and synthesizing DNA before they were arrested at the 4C DNA population.

Therefore we anticipated some recovery from lethality after 9L spheroids were treated with SHM. The CFE assay of spheroid cells in each fraction elutriated 24 hr after treatment with 3.0 μg/ml SHM showed that PEs of 2C DNA-enriched cells were significantly higher ($P > 0.01$) than PEs of 4C DNA-enriched cells, whereas corresponding cell populations from untreated spheroid cells had PEs of approximately 50% throughout the incubation period. The PE of cells with 2C DNA was close to the PE of untreated cells, and the PE of cells with 4C DNA—presumably cells that were cycling at the time of treatment—was similar to the PE of monolayer cells incubated for 24 hr after treatment with SHM [4].

Intact 9L spheroids exposed to 3 μg/ml of SHM had a 60–70% kill if assayed 1–6 hr after treatment but had only a 45% cell kill if assayed 24 hr after treatment (Fig. 2). The time-dependent recovery of spheroids treated with SHM at various doses (1.5–6.0 μg/ml) is probably the result of the ability of non-cycling cells to repair the damage even though it is not prominent, as found for 9L cells in monolayer culture [5]. Thus this recovery may or may not be due to recovery from the PLD observed in 9L monolayer cells because spheroids were not placed in depleted medium or serum-free medium. However, it is reasonable to assume that SHM killed more cells in monolayer culture than cells in spheroids or *in situ* tumors because of the apparent resistance to SHM of non-cycling cells in spheroids and tumors. In this sense, SHM is an alkylator that is preferentially effective against cycling cells. We cannot explain the apparent additional cell kill observed at 6 hr compared to 1 hr, but it is probably not the result of diffusion limitations because the drug loses its activity in cell culture medium within 30 min at 37°C and pH 7.3. This presumes, of course, that altered physiologic conditions within the spheroid do

not significantly prolong the half-life of SHM decay.

At low (μM) concentrations SHM was cytotoxic to 9L monolayer cells [4, 8] but had almost no cytotoxic effect against 9L tumor-bearing rats [4]. While we do not rule out the possibility that this could be the result of differences in the ability to deliver the drug, our observations suggest that the relative resistance of non-cycling cells to SHM plays an important role in the biological activity of SHM.

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REFERENCES

1. PLOWMAN J, LAKINGS DB, OWENS ES, 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. 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.
5. 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.
6. 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.
7. HOSHINO T, DEEN DF, WILLIAMS ME, SANO Y. Differential response of elutriated 9L cells to BCNU treatment. *Cancer Res* 1981, **41**, 4404–4407.
8. DEEN DF, BARTLE RM, WILLIAMS ME. Response of cultured 9L cells to spirohydantoin mustard and X-rays. *Int J Radiat Oncol Biol Phys* 1979, **5**, 1663–1667.
9. GRABSKE RJ, LAKE S, GLEDHILL BL, MEISTRICH ML. Centrifugal elutriation: separation of spermatogenic cells on the basis of sedimentation velocity. *J Cell Physiol* 1975, **86**, 177–189.
10. DEAN PN. Simplified methods of analyzing DNA distributions from perturbed cell populations. *Cell Tissue Kinet* 1980, **13**, 672.
11. WEIZSAECKER M, HOSHINO T, KOBAYASHI S. Effect of tritiated thymidine on the kinetics and viability of 9L cells *in vitro*. *Cell Tissue Kinet* 1981, **14**, 575–580.