Anti-Cancer Drugs, 6, pp. 115–123

Comparison of sulforhodamine B, tetrazolium and clonogenic assays for *in vitro* radiosensitivity testing in human ovarian cell lines

Geneviève Griffon,[1] Jean-Louis Merlin[1] and Christian Marchal[2]

[1]Laboratoire de Recherche en Oncologie and [2]Service de Radiothérapie, Centre Alexis Vautrin, 54511 Vandœuvre-les-Nancy, France. Tel: (+33) 83 59 85 06; Fax: (+33) 83 44 60 71.

The radiation sensitivity of six human ovarian tumor cell lines was evaluated using sulforhodamine B (SRB), tetrazolium (MTT) and clonogenic assays. Radiobiological parameters calculated from a linear quadratic model (SF_2, α, β) as well as from a single-hit multitarget model (D_0, D_q, n) and from the area under the dose–response curve (mean inactivation dose; MID) were compared. If the values deduced from MTT experiments were statistically comparable to those obtained from clonogenic assays, significant differences were observed between SRB and the two other assays that concerned the results achieved with the highest radiation doses tested (6–8 Gy), yielding a surviving fraction of approximately 20%. In addition, the intra- and inter-experimental variation of SRB dramatically increased within this range of radiation doses. However, up to 6 Gy, the SRB assay proved to be statistically comparable to MTT and clonogenic assays, and allowed the calculation of SF_2, α and MID radiobiological parameters.

Key words: Clonogenic assay, MTT assay, ovarian tumour, radiosensitivity, SRB assay.

Introduction

Several *in vitro* assays methodologies have been used to evaluate the cytotoxicity of therapeutic procedures in cancer research. Radiation sensitivity has been assessed in numerous cell lines over the past 20 years using clonogenic assays, which has generally been considered as the optimal method since its original description in 1955.[1] Short- and intermediate-term non-clonogenic assays, validated from the assessment of chemosensitivity, were often considered to be inadequate to measure radiation sensitivity because of the short duration of the assays. Following a dose of radiation, cells destined to die undergo one or more cell divisions and, during the time interval, lethally irradiated cells appear to function normally with respect to their metabolism and the maintenance of intact cell structure.[2] If non-clonogenic assays such as [^{3}H]thymidine incorporation,[3] fluorescence-based microtiter plate assays[4] or reduction of various dyes[5] are used for radiation survival assessment, it is important that adequate time elapses following the radiation exposure before performing the assay. However, several pitfalls remain in the clonogenic assay since some cell lines do not form colonies or have a low plating efficiency.[6,7]

In recent years, recognition of these potential problems has spurred development of several alternative assays including the microtetrazolium assay (MTT), which quantifies metabolically viable cells through their ability to reduce a soluble yellow tetrazolium salt to insoluble blue–purple formazan crystals.[5] The crystals are produced by the mitochondrial succinate deshydrogenase enzyme, and can be dissolved and quantified by measuring the absorbance of the resulting solution. Several modifications of the original method[5] have been described and largely used to measure chemosensitivity in human tumor cell lines[6,8] as well as fresh human leukemia cells.[9] The use of the MTT assay in assessing the response of cells to non-chemotherapeutic treatments such as ionizing radiation[2,10–12] or photodynamic therapy[13] has also been reported. This assay has the advantage of being rapid, precise and semi-automatable. However, a few problems exist, since the cell lines may differ in their capacity to reduce the dye,[14] the absorbance of the solution is only proportional to the number of viable cells within certain limits[2,3,8,10] and at low cell densities, no significant difference in absorbance can be measured with the blanks.[15] Furthermore, the results may vary under a number of conditions such as MTT concentration and incubation time, thus leading to some large intra- and inter-assay variations.[7,10]

Financial support was obtained from Alexis Vautrin Cancer Center Research Fund and from the French 'Ligue Nationale Contre le Cancer, Comité départemental de Meurthe et Moselle'.

Correspondence to G Griffon

More recently, the US National Cancer Institute (NCI) adopted the use of the sulforhodamine B (SRB) protein staining for *in vitro* chemosensitivity testing, which quantifies viable cells by measuring their total protein content.[15–18] The SRB assay appeared to be more sensitive than the MTT assay, with a better linearity with cell number and higher reproducibility.[15] To our knowledge, very few authors[19] have reported the use of the SRB assay in assessing the response of cells to ionizing radiation.

In this paper, we report a comparison between the SRB, MTT and clonogenic assays with regard to the response of six ovarian tumor cell lines to ionizing radiation.

Materials and methods

Cell lines

Six human ovarian cell lines were studied. SKOV-3, NIH-OVCAR3, HEY and HOC7 lines were provided generously by Dr C Dittrich (Department of Internal Medicine I, Division of Oncology, University of Vienna, Austria), and A2780 and its multidrug resistant variant $A2780^{AD}$ lines by Professor EA De Bruijn (Laboratory of Cancer Research and Clinical Oncology, University of Wilrijk, Belgium).

All these cell lines were continuously maintained as monolayer cultures in RPMI 1640 medium (Gibco, Eragny, France) supplemented with 10% inactivated fetal calf serum (Dutscher, Brumath, France), penicillin (500 UI/ml), gentamycin (5 μg/ml), hydrocortisone (0.5 mg/ml), transferrin (2.5 μg/ml), epidermal growth factor (10 μg/ml), insulin (10 mg/ml) and glutamine (0.3 mg/ml). Cell cultures were passaged every 4 days to ensure exponential growth.

MTT assays

Cells were harvested from exponential phase culture by trypsinization, counted and plated in 96-well microtiter plates. Optimal seeding densities of each cell line were determined to ensure adequate absorbance reading in control wells during a 10 day assay. A concentration of 1500 cells/ml (300 cells/well) was found suitable and used for all cell lines. Before performing growth-inhibition assays, we examined the linearity of the MTT assay with increasing numbers of seeded cells (0–800 cells/well). Before being irradiated, the cells were incubated for 8 h to ensure their attachment to the bottom of the wells. Following treatment, assays were run after 10 days corresponding to at least six cell-doubling times.[2] Culture medium was removed from the wells every 3 days and replaced by 200 μl of fresh medium. Following this incubation period, the effect of irradiation was estimated using the tetrazolium salt MTT [3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma, St Quentin Fallavier, France) to measure the metabolic activity of tumor cells.[5] MTT was dissolved in phosphate buffered saline (PBS), filter-sterilized and stored at 4°C. MTT (50 μl of a 0.5% solution) was added to each well and incubated for 3 h at 37°C to allow metabolization. The formazan crystals were dissolved by adding 50 μl of 25% sodium dodecylsulfate solution (Sigma). Plates were shaken for 5 min on a plate shaker (Dynatech, Guyancourt, France) to ensure an adequate solubilization. Absorbance was measured at 540 nm on a MCC/340 Titertek Multiskan (Flow, Les Ulis, France).

SRB assays

The SRB assay was performed according to the method of Skehan *et al.*[18] The methods of plating and incubation were identical to the MTT assay. We examined the linearity of the SRB assay under the same conditions as the MTT assay. Following the 10 day incubation, the cells were washed with PBS in order to eliminate the culture medium and were fixed by means of protein precipitation with 50% trichloroacetic acid (TCA) at 4°C (50 μl/well, final concentration 10%) for 1 h. After five washings with tap water to remove TCA, low molecular weight metabolites and cellular fragments, the cells were stained for at least 15 min with 0.4% SRB solution (Aldrich-Chimie, St Quentin Fallavier, France) dissolved in 1% acetic acid (50 μl/well). At the end of the staining period, the monolayers were rinsed four times with 1% acetic acid to remove unbound dye. The plates were air-dried until no standing moisture was visible. Protein bound stain was solubilized with 150 μl of 10 mmol/l unbuffered Tris base [tris (hydroxymethyl) aminomethane] (Merck, Darmstadt, Germany) (pH 10.5). The optical density was read at 540 nm on a MCC/340 Titertek Multiskan.

For both colorimetric assays, 18 wells were used for each radiation dose and the radiosensitivity assay was assessed in triplicate for each cell line. In both assays, background optical densities were measured in wells incubated with growth medium without cells. Results were expressed as relative percentages of absorbance as compared with untreated controls.

Clonogenic assays

Clonogenic assays were performed using a technique previously described[20] and adapted from Hamburger and Salmon.[21] Briefly, 5×10^3 to 5×10^4 cells/ml were suspended in 1 ml of 0.3% molten agar (Bacto agar; Difco, Detroit, MI) containing enriched medium and 10% FCS. The cells were then plated in dishes (35 mm diameter) over a 1 ml 0.5% agar underlayer containing the same medium. Cultures were irradiated after mixing in agar and were then incubated at 37°C with 5% CO_2 in air for 14 days. Only colonies consisting of 50 cells or more were counted and survival was calculated by comparing the number of colonies in irradiated dishes with untreated control dishes.

Irradiation procedure

All irradiations were performed using a Theratron 780C ^{60}Co unit (Theratronics, Ottawa, Canada) operating at 1.25 MeV. Cells were irradiated at a dose rate of 5 Gy/min at room temperature. Doses ranged from 0 to 8 Gy (0, 2, 4, 6 and 8 Gy). Control plates were removed from the incubator for the same period of time, but were not irradiated.

Dose–response curve analysis

Dose–response curves of each cell line obtained from three assays were fitted using the linear quadratic (LQ) model

$$SF = e^{-\alpha D - \beta D^2}$$

and the parameters α, β, the calculated surviving fraction (SF) at 2 Gy (SF_2)[22] as well as the mean inactivation dose (MID)[23] were deduced. MID was obtained by integration between dose 0 to infinity in linear coordinates and Fertil *et al.*[23] advocated it as a measure of intrinsic radiation sensitivity of human cells. The linear component α (initial slope of this dose–survival relationship) represents single-hit killing kinetics and dominates the radiation response at low doses (D). The quadratic component of cell killing, β, causes the curve to bend at higher doses.

The parameters D_0 and n of the single-hit multitarget model were also computed.

$$S/S_0 = 1 - (1 - e^{D/D_0})^n$$

D_0 is the dose that gives an average of one hit per target. A dose of D_0 Gy reduces survival from 1 to 0.37. The extrapolation number, n, is determined by extrapolating the final linear slope back to the ordinate and designates the number of targets. D_q is the 'quasi-threshold' dose and measures the size of the shoulder.

Statistical analysis

An ANOVA test with Scheffé's procedure was used to investigate whether the parameters of three dose–response curves of each cell line obtained from the three assays were significantly different. A significance level of $p \leq 0.05$ was used throughout.

Results

Relationship between MTT and SRB absorbance and cell number

For each cell line, cells were distributed into 96-well plates in serial dilutions from 0 to 800 cells/well and incubated for 10 days at 37°C corresponding to at least six cell-doubling times which were necessary to evaluate responsiveness to radiation doses. After this period, MTT and SRB assays were performed. The relationship between absorbance and the number of cells plated was linear (Figure 1a) from 0 to 500 cells/well (except the NIH-OVCAR3 and HEY cell lines for which the linearity was only obtained between 0 and 400 cells/well) either with MTT (mean r = 0.961; range 0.938–0.975) or SRB assay (mean r = 0.981; range 0.980–0.997). The SRB assay was found more sensitive than the MTT assay, the absorbance values being generally two to three times higher, depending on the cell line tested (Figure 1a and b). Above 400 or 500 cells/well, depending on the cell line, after 10 days, the cells in monolayer culture had almost become confluent and died, thus resulting in a decrease in absorbance.

Dose–response curve characteristics

Radiation dose–response curves derived from the three assays performed with each cell line (except NIH-OVCAR3 and SKOV-3 cell lines which did not grow as colonies) are illustrated in Figure 2. Whereas the curves obtained from MTT and clonogenic assays were quite comparable, those estab-

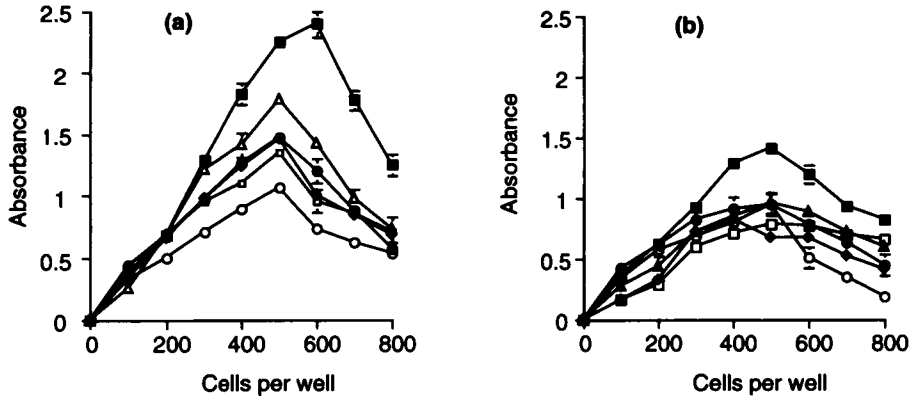

Figure 1. Calibration curve for MTT and SRB absorbance versus cell number for HOC-7 (■), NIH-OVCAR3 (◆), HEY (●), SKOV-3 (△), A2780^{AD} (○) and A2780 (□). Cells were plated at the indicated cell densities and after a 10 day period both SRB (a) and MTT (b) assays were read at 540 nm. Each point represents mean and SD of a mean of three replicates (if not indicated, error bar was within size of symbol).

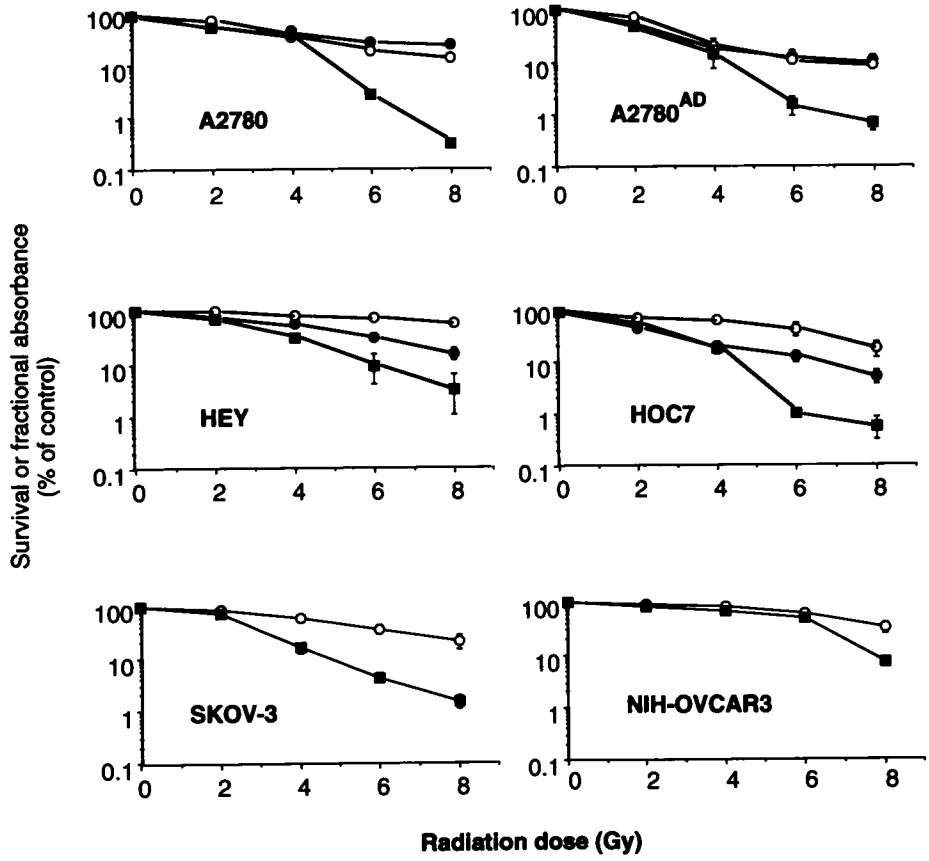

Figure 2. Dose–response curves for irradiation using clonogenic (●), MTT (○) and SRB (■) assays for six different cell lines. Individual points represent data of a minimum of three experiments. (Bars: standard errors of mean; if not indicated, error bars were within size of symbol.)

lished from the SRB assay showed a sharper downward part at high doses (from 4 Gy for the HEY, HOC-7, A2780, A2780^{AD} and SKOV-3 cell lines; 6 Gy for the NIH-OVCAR3 cell line).

Comparison of radiosensitivity parameters obtained by the SRB, MTT and clonogenic assays

The values of dose–response curve parameters (SF_2, α, β, MID, D_0, D_q and n) calculated from the three assays are listed in Table 1 and compared in Table 2. Among the two non-clonogenic assays, only some radiosensitivity parameter values obtained from the MTT assay were significantly different ($p \leq 0.01$) with those obtained from the clonogenic assay which were lower in HOC-7 cells (SF_2, α, MID and D_q) and HEY cells (MID and D_0). It was shown that the A2780 cell line presented significant differences in several radiosensitivity parameters (α, MID, D_q and n), and in two cases, HEY (SF_2) and A2780^{AD} (D_0), parameter values obtained from SRB and clonogenic assays were not significantly different. On the other hand, a greater statistical difference was observed (Table 2) between the radiosensitivity parameters calculated from SRB and MTT assays than those calculated from both colorimetric and clonogenic assays. Only the radiosensitivity parameters in A2780^{AD} cells were similar whatever the method, except D_0. In contrast, most parameters were significantly different in A2780 cells. However, if the highest radiation dose (8 Gy) was not considered in the SRB assay, no significant difference was observed between the results obtained from the three assays (Table 2).

Intra- and inter-experimental variations

The intra- and inter-experimental coefficients of variation (CV) were calculated for each cell line, for each culture method and for each radiation dose from the optical densities (MTT and SRB assays) or number of colonies formed (clonogenic assay). Repeated experiments were performed on the same culture to evaluate the intra-experimental variations. Three assays were performed on the six cell lines. Heterogeneity between the cultures gave a CV of 8.3% (range 3.8–15.8), 7.8% (range

Table 1. Radiosensitivity parameters (mean ± SEM) obtained from clonogenic (CA), MTT and SRB assays: all data sets are means of three separate experiments

Radiosensitivity parameters	Assay	Cell lines					
		A2780	A2780^{AD}	HEY	HOC-7	OVCAR-3	SKOV-3
SF_2	CA	0.77 ± 0.01	0.53 ± 0.03	0.89 ± 0.02	0.60 ± 0.01		
	MTT	0.72 ± 0.01	0.51 ± 0.02	0.97 ± 0.01	0.91 ± 0.02	0.93 ± 0.01	0.82 ± 0.04
	SRB	0.75 ± 0.02	0.37 ± 0.10	0.76 ± 0.01	0.57 ± 0.04	0.87 ± 0.01	0.51 ± 0.02
α	CA	0.13 ± 0.01	0.32 ± 0.03	0.04 ± 0.01	0.25 ± 0.01		
(Gy^{-1})	MTT	0.15 ± 0.02	0.33 ± 0.02	0.01 ± 0.01	0.01 ± 0.01	0.0	0.07 ± 0.02
	SRB	0.01 ± 0.01	0.44 ± 0.18	0.12 ± 0.3	0.19 ± 0.09	0.0	0.27 ± 0.04
β	CA	0.001	0.0	0.01	0.002		
(Gy^{-2})	MTT	0.004	0.0	0.01	0.02	0.02	0.02
	SRB	0.06	0.03	0.02	0.04	0.03	0.03
MID	CA	7.7 ± 0.8	3.1 ± 0.4	7.2 ± 0.35	3.8 ± 0.3		
(Gy)	MTT	5.4 ± 0.6	3.0 ± 0.3	14.4 ± 1.2	6.6 ± 0.7	6.9 ± 0.7	5.3 ± 0.6
	SRB	3.4 ± 0.1	1.9 ± 0.4	4.9 ± 1.8	2.7 ± 0.2	4.7 ± 0.2	2.5 ± 0.06
D_0	CA	7.5 ± 0.9	3.1 ± 0.4	6.1 ± 0.3	3.7 ± 0.4		
(Gy)	MTT	5.0 ± 0.8	3.0 ± 0.3	19.4 ± 3.4	5.0 ± 1.3	5.2 ± 1.2	3.6 ± 0.7
	SRB	1.3 ± 0.02	1.3 ± 0.1	3.9 ± 1.3	1.4 ± 0.2	2.3 ± 0.1	1.5 ± 0.1
D_q	CA	0.1 ± 0.0	0.0	2.0 ± 0.4	1.1 ± 1.1		
(Gy)	MTT	0.6 ± 0.4	0.03 ± 0.03	2.0 ± 0.2	2.3 ± 0.3	2.6 ± 0.0	2.0 ± 0.5
	SRB	2.6 ± 0.04	1.1 ± 0.7	1.4 ± 0.7	1.9 ± 0.5	2.6 ± 0.0	1.5 ± 0.2
n	CA	1.0	1.0	1.4	1.1		
	MTT	1.2	1.0	1.1	1.8	1.7	1.7
	SRB	7.6	2.3	1.4	5.0	3.0	3.0

Table 2. Comparison of radiosensitivity parameters calculated from the dose–response curves using the ANOVA test with Scheffé's procedure (these curves were fitted from the data of clonogenic, MTT and SRB assays)

Radiosensitivity parameters	Cell lines					
	HEY	HOC-7	A2780	A2780^{AD}	SKOV-3	NIH-OVCAR3
SF_2	SRB–CA *0.011[b] SRB–MTT 0.019	SRB–MTT <0.01 MTT–CA <0.01	NS[a]	NS	SRB–MTT *<0.01	SRB–MTT *<0.01
α (Gy^{-1})	NS	MTT–CA <0.01	SRB–CA *<0.01 SRB–MTT *<0.01	NS	SRB–MTT *<0.01	NS
β(Gy^{-2})	NS	NS	SRB–MTT *<0.01 MTT–CA *<0.01	NS	SRB–MTT *0.04	SRB–MTT *<0.01
MID (Gy)	SRB–MTT <0.01 MTT–CA <0.01	SRB–MTT <0.01 MTT–CA 0.01	SRB–CA *0.029	NS *<0.01	SRB–MTT *<0.01	SRB– MTT *<0.01
D_0 (Gy)	SRB–MTT <0.01 MTT–CA 0.01	SRB–MTT *0.02	SRB–CA *<0.01 SRB–MTT *<0.01	SRB–CA <0.01 SRB– MTT <0.01	SRB–MTT *0.01	SRB–MT *<0.01
D_q (Gy)	NS	MTT–CA <0.01	SRB– CA *<0.01 SRB–MTT *<0.01	NS	NS	NS
n	NS	NS	SRB–CA *<0.01 SRB–MTT *<0.01	NS	NS	NS

[a] Non-significant difference between values of the parameters ($p > 0.05$).
[b] $p \leq 0.05$, values of the parameters significantly different.
* When the dose of 8 Gy was suppressed, no significant difference was observed between the confered assays.
a = CA = clonogenic assay

1.1–26.6%) and 14.0% (range 10–21%) for the MTT, SRB and clonogenic assays, respectively. We observed that the more the radiation dose increased, the higher the CV values were, whatever the assay. Lower variations were observed for SRB assays than the other assays when the radiation dose was below 8 Gy.

Repeated assays were performed to evaluate inter-experimental variations. Three to eight repeated assays were performed on the six cell lines. The mean values of the inter-experimental CV were 18.0% (range 14–22%), 17.0% (range 4.5–46%) and 20% (range 12.5–27%) for MTT, SRB and clonogenic assays, respectively. At 8 Gy, important inter-experimental variations were observed, as already noted for intra-experimental variations. The mean values of inter-exerimental CV obtained from MTT and clonogenic assays, where the highest values did not correspond to the highest radiation doses, were found more heterogeneous than for SRB assays, in which a progressive increase was observed with the radiation dose.

Discussion

Cell survival after irradiation is traditionally measured using clonogenic assays. However, limitations include the inability to measure survival in cells which do not grow as colonies or have low plating efficiency. Some non-clonogenic assays were proposed in order to palliate these inconvenients. The MTT assay quantifies metabolically viable cells by their ability to reduce the tetrazolium derivative MTT, whereas the SRB assay quantifies viable cells by measuring their total protein content. Using both assays, optimal seeding cell number and assay duration required elucidation for each cell

line. It is obviously essential that enough time is allowed for cell death and that all treatment groups, particularly control cells, remain in exponential growth up to the end of assay. Price and McMillan[10] found that the actual values obtained for a growth assay vary considerably with the assay conditions and Carmichael *et al.*,[2] using established cell lines, showed that delaying analysis until control cells had undergone at least six doubling times gave results comparable to clonogenic assay. To our knowledge, the use of the SRB assay in assessing the response of cells to ionizing radiation has been studied only by van Geel *et al.*,[19] who used SRB to stain colonies. In our case, the ovarian tumor cell lines studied had a doubling time of about 34 h and the time needed to obtain at least six doubling times was estimated as 10 days. Thus, the cellular density of each cell line (300 cells/well) has been evaluated from the relationship between cell number and SRB and MTT absorbance.

Studies concerning the evaluation of radiosensitivity parameters using the MTT assay were reported in Chinese hamster V79, two human lung tumor cell lines[2] and in primary culture obtained from malignant glioma.[11] Nevertheless, our results were consistent with those reported by Rofstad *et al.*[24,25] in ovarian biopsies and cell lines using clonogenic assays. Some authors[2,10–12] found a good agreement between radiation dose–response data for cell lines obtained by MTT and clonogenic assays. In our study, some parameters obtained from MTT assays under our experimental conditions were found higher than those obtained from clonogenic assays. According to Ramsay's results,[11] this could be explained by the fact that the MTT assay measured variations in total cell number, while the clonogenic assay measured the survival of clonogens which may represent less than 1% of the total cell number. However, there was reasonable qualitative agreement between the assays.[11] Carmichael *et al.*[2] explained this phenomenon by the possibility that deshydrogenase activity was maintained for some time after the cells had been killed. This factor could certainly result in an artifactual shoulder on the dose–response curve affecting the parameters related to the low-dose region.

The SRB protein stain assay was used usually for *in vitro* chemosensitivity testing.[3,26] Van Geel *et al.*[19] used this assay associated with the clonogenic assay for the evaluation of the radiosensitivity of fibroblasts obtained from skin biopsies. In staining colonies by the SRB, a good correlation was observed between the number of colonies counted and SRB-related absorbance. In the present experiments, numerous radiosensitivity parameters calculated from SRB assays were lower than those obtained by clonogenic assays (Table 1); however, not all were significantly different. This observation may explain the wide difference seen between both colorimetric assays. Nevertheless, when the radiation dose of 8 Gy was not considered for the SRB assay, no significant difference remained between the three methods for every cell line except HOC-7, in which a better correlation between the SRB and clonogenic assays was observed, whereas the parameters SF2, α and MID obtained from the MTT assay differed with those obtained from the other assays. This could be elucidated by Carmichael's explanation[2] about the MTT assay. Rubinstein *et al.*[17] showed that if screening data analyses are limited to the use of IC_{50} values, the MTT and SRB assays gave comparable results. Indeed, they compared the chemosensitivity of 38 cell lines to 197 compounds using the MTT and SRB assays, and only found discrepancies between the two assays for a small subset of compounds. In addition, the phenomenon was restricted to the portion of the dose–response curve below 10% of relative growth. Then, if values relating to high levels of cell kill were used, such as IC_{90} values, attention should be paid to the discrepancies between the two assays. If a relatively high radiation dose such as 8 Gy was assimilated to IC_{90} killing a high fraction of cells, the SRB assay did not give results comparable with the MTT and clonogenic assays. When exposed to high radiation doses, some viable cells might be more fragile, less adhesive to the support and be lost during the washing steps which are more numerous in the SRB assay than in the MTT assay.

This might also explain why intra-experimental CV values measured from SRB assay, being about 3% up to 6 Gy, shifted to about 27% at 8 Gy. The analysis of inter-experimental variations also revealed this limit as well as the analysis of radiosensitivity parameters, where no significant difference was observed when the SRB assay was limited to doses, up to 4 or 6 Gy, corresponding to a surviving fraction of approximately 20%. Within this range of doses, the SRB assay was highly reproducible, confirming what has already been demonstrated in chemosensitivity experiments.[15,18]

The SRB assay proved useful to measure the *in vitro* radiosensitivity of human ovarian tumor cells. It presents the same advantages over clonogenic assays as the MTT assay: both colorimetric assays are related to the total cell number, while the clonogenic assay measures the survival of clonogens.[11]

The semi-automatation which is possible with colorimetric assays made the experiment less tedious than the clonogenic assays. However, the duration of both the MTT and SRB assays should be adapted to the doubling time of the cell population analyzed. It remains to be established whether it would be necessary to adjust the assay time to optimize the radiation response data obtainable from tumor cell suspensions.[2,12]

The SRB assay offers major practical and biological advantages over the MTT assay. Notably, tetrazolium assays involve a timed step in which cells are incubated with tetrazolium and the number of viable cells is estimated from the amount of formazan produced. Therefore, the reading of plates should be immediate because of the instability of the staining obtained after reduction of the tetrazolium salt. On the contrary, SRB staining is stable and plates can be stored for several weeks up to several months.[15] In addition, the SRB assay is independent of intermediary metabolism and of a variety of metabolic conditions (depletion of glucose, variations in pH) which might alter the production of formazan from tetrazolium.[17] Furthermore, the SRB assay provided a better linearity with cell number than the MTT assay, a better sensitivity due to absorbance values (generally two to three times higher), and better reproducibility and repeatability than the MTT and clonogenic assays.

If discrepancies appeared between SRB and both MTT and clonogenic assays, they seem limited to high levels of growth-inhibitory effects achieved with relatively high irradiation doses. This has limited consequences since the parameters α, SF_2 and MID depend essentially on the initial part of dose–response curve.[27]

In summary, this study reported that the SRB assay can be used under well-defined experimental conditions to give qualitatively similar results to the MTT and clonogenic assays for radiosensitivity testing but only at low radiation doses. Because of its large linearity range, the SRB assay could be suitable to study the radiosensitivity of subconfluent monolayers and three-dimensional models, such as multilayer cell clusters containing large amounts of cells,[18,28] three-dimensional histocultures[29] and spheroids, whch represent available models in radiobiology.[9]

Acknowledgments

The authors would like to thank Dr PE Pizao (Sao Paulo, Brazil) for helpful discussion concerning the SRB assay. This study was performed under the auspices of the EORTC Preclinical Therapeutics Models Group and presented at the 36th Annual Scientific Meeting of the American Society for Therapeutic Radiology and Oncology (ASTRO) in San Francisco, October 2–7, 1994.

References

1. Puck TT, Marcus P. A rapid method of viable cell titration and clone production with HeLa cells in tissue culture: the use of X-irradiated cells to supply conditioning factors. *Proc Natl Acad Sci USA.* 1955; **41**: 432–7.
2. Carmichael J, Degraff WG, Gazdar AF, *et al.* Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of radiosensitivity. *Cancer Res* 1987; **47**: 943–6.
3. Twentyman PR, Luscombe M. A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. *Br J Cancer* 1987; **56**: 279–85.
4. Begg AC, Mooren E. Rapid fluorescence-based assay for radiosensitivity and chemosensitivity testing in mammalian cells *in vitro*. *Cancer Res* 1989; **49**: 565–9.
5. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983; **65**: 55–63.
6. Von Hoff DD. He's not going to talk about *in vitro* predictive assays again, is he? *J Natl Cancer Inst* 1990; **82**: 96–101.
7. Park IJ, Kramer BS, Steinberg SM, *et al.* Chemosensitivity testing of human colorectal carcinoma cell lines using a tetrazolium based colorimetric assay. *Cancer Res* 1987; **47**: 5875–9.
8. Carmichael J, Degraff WG, Gazdar AF, *et al.* Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res* 1987; **47**: 936–42.
9. Twentyman PR, Fox NE, Rees JKH. Chemosensitivity testing of fresh leukemia cells using the MTT colorimetric assay. *Br J Haematol* 1989; **71**: 19–24.
10. Price P, McMillan TJ. Use of the tetrazolium assay in measuring the response of human tumor cells to ionizing radiation. *Cancer Res* 1990; **50**: 1392–6.
11. Ramsay J, Ward R, Bleehen NM. Radiosensitivity testing of human malignant gliomas. *Int J Radiat Oncol Biol Phys* 1992; **24**: 675–80.
12. Wasserman TH, Twentyman PR. Use of the colorimetric microtiter (MTT) assay in determining the radiosensitivity of cells from murine solid tumors. *Int J Radiat Oncol Biol Phys* 1988; **15**: 699–702.
13. Merlin JL, Azzi S, Lignon D, *et al.* MTT assays allow quick and reliable measurement of the response of human tumour cells to photodynamic therapy. *Eur J Cancer* 1992; **28A**: 1452–8.
14. Scuidiero DA, Shoemaker RH, Paull KD, *et al.* Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. *Cancer Res* 1988; **48**: 4827–33.
15. Keepers PY, Pizao PE, Peters GJ, *et al.* Comparison of the sulforhodamine B protein and tetrazolium (MTT) assays for *in vitro* chemosensitivity testing. *Eur J Cancer* 1991; **7**: 897–900.
16. Perez RP, Godwin AK, Handel LM, *et al.* A comparison of clonogenic, microtetrazolium and sulforhodamine B as-

says for determination of cisplatin cytotoxicity in human ovarian carcinoma cell lines. *Eur J Cancer* 1993; **29A**: 395–9.
17. Rubinstein LV, Shoemaker RH, Paull KD, *et al.* Comparison of *in vitro* anticancer-drug-screening data generated with a tetrazolium assay versus a protein assay against a diverse panel of human tumor cell lines. *J Natl Cancer Inst* 1990; **82**: 1113–8.
18. Skehan P, Storeng R, Scudiero D, *et al.* New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst* 1990; **82**: 1107–12.
19. Van Geel CAJF, Van den Berg AP, Van den Aardweg GJMJ. Good correlation between the colorimetric SRB assay and the clonogenic assay for cellular radiosensitivity in different cell types. In: *European Society for Radiation Biology, European Society for Hyperthermic Oncology* 1994: 49.
20. Merlin JL, Chastagner P, Marchal C, *et al.* *In vitro* combination of high dose busulfan with radiotherapy on medulloblastoma cells: additive effect without potentiation. *Anti-Cancer Drugs* 1991; **2**: 465–8.
21. Hamburger AW, Salmon SE. Primary bioassay of human tumor stem cells. *Science* 1977; **197**: 461–3.
22. Fertil B, Malaise EP. Inherent cellular radiation as a basic concept for human tumor radiotherapy. *Int J Radiat Oncol Biol Phys* 1981; **7**: 621–9.
23. Fertil B, Dertinger H, Courdi A, *et al.* Mean in activation dose: a useful concept for intercomparison of human cell survival curves. *Radiat Res* 1984; **99**: 73–84.
24. Rofstad EK, Sutherland RM. Radiation sensitivity of human ovarian carcinoma cell lines *in vitro*: effects of growth factors and hormones, basement membrane, and intercellular contact. *Int J Radiat Oncol Biol Phys* 1988; **5**: 921–9.
25. Rofstad EK, Wahl A, Brustad T. Radiation sensitivity *in vitro* of cells isolated from human tumor surgical specimens. *Cancer Res* 1987; **47**: 106–10.
26. Twentyman PR, Walls GA, Wright KA. The response of tumor cells to radiation and cytotoxic drugs—a comparison of clonogenic and isotope uptake assays. *Br J Cancer* 1984; **50**: 625–31.
27. Fertil B, Malaise EP. Intrinsic radiosensitivity of human cell lines is correlated with radioresponsiveness of human tumors. Analysis of 101 published survival curves. *Int J Radiat Oncol Biol Phys* 1985; **9**: 1699–707.
28. Pizao PE, Lyaruu DM, Peters GJ, *et al.* Growth, morphology and chemosensitivity studies on postconfluent cells cultured in 'V'-bottomed microtiter plates. *Br J Cancer* 1992; **66**: 660–5.
29. Hoffman RM. Three-dimensional histocultures: origins and applications in cancer research. *Cancer Cells* 1991; **3**: 86–92.

(Received 17 October 1994; accepted 10 November 1994)