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Acknowledgements——This work was supported by the Cancer Society of New Zealand, by its Auckland Division, and by the Health Research Council of New Zealand. The authors would like to thank the patients who allowed samples of their melanomas to be used for this research project.

Pergamon

European Journal of Cancer Vol. 30A, No. 9, pp. 1376–1382, 1994
Elsevier Science Ltd
Printed in Great Britain
0959–8049/94 $7.00+0.00

0959-8049(94)00243-6

Measurement of Radiation Survival Using the MTT Assay

A. Slavotinek, T.J. McMillan and C.M. Steel

There is increasing interest in the development of rapid assays of radiosensitivity which can be used on clinical specimens. Unfortunately, the measurement of radiation survival using clonogenic assays, which are the established standard, can be difficult and time consuming. We have used the MTT assay to measure the radiation survival of four lymphoblastoid cell lines with low plating efficiencies. We measured surviving fractions both when the irradiated cells had regained exponential growth and when the non-irradiated cells had undergone four or more doublings. The results were compared to surviving fractions measured by clonogenic assay. We found both methods could be used successfully to rank the cell lines in order of radiosensitivity. However, cells exposed to the higher radiation doses in the MTT assay did not always regain exponential growth, limiting the dose range for which the assay was useful. We also found the best correlation between the two assays was sometimes obtained by using the MTT surviving fractions from different days for different radiation dose levels. Thus, although the MTT assay can be used to measure radiation survival in relation to other cell lines, its use can be complicated by restrictions on radiation dose ranges and difficulties with data interpretation.

Key words: MTT assay, clonogenic assay, radiation survival, lymphoblastoid cell lines
Eur J Cancer, Vol. 30A, No. 9, pp. 1376–1382, 1994

INTRODUCTION

THE RADIATION survival of cells has traditionally been measured by clonogenic assay [1, 2]. However, such assays require reliable cell growth in culture media, and can be difficult and time consuming to perform in cell lines with low plating efficiencies [3]. The search for alternative methods to measure radiation survival has resulted in several evaluations of the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay for this purpose [4–7]. The MTT assay quantifies the ability of viable cells to reduce a yellow tetrazolium salt to a purple formazan crystal using the mitochondrial enzyme, succinate dehydrogenase [8]. The optical density of the solution resulting from the solubilisation of the formazan precipitate with an organic solvent can be detected using a multiwell spectrophotometer [9]. The MTT assay is thus semi-automatable and rapid, and has been used successfully to test the chemosensitivity of established cell lines [10, 11] and fresh tumor samples [12–14].

The use of the MTT assay for the measurement of radiation

survival has produced conflicting results. Comparable results to clonogenic assays have been obtained both when the surviving fractions of tumour cell lines were measured after 4–7 cell doubling times [4], and after the irradiated cells had regained exponential growth [6]. Other research comparing the two assays has found that the surviving fractions measured by MTT assay were higher than those from clonogenic assays [5, 7]. However, Ramsay and colleagues concluded that the MTT assay increased the proportion of successful tests, and that they were of shorter duration [7].

The MTT assay has mostly been used to measure radiation survival of adherent tumour cell lines to minimise the potential cell loss that can occur with the aspiration of medium before the addition of organic solvent or before refeeding [4]. In these experiments, we have used the MTT assay and a clonogenic assay to measure radiation survival in lymphoblastoid cell lines that grow in suspension to investigate further the potential of this assay for use in cell lines with low plating efficiencies.

MATERIALS AND METHODS

Cell lines and culture medium

The cell lines used were K562, a myeloid erythro-leukaemia cell line; LICR-LON, a B-cell plasma leukaemia cell line; DEW1, a cell line derived from a patient with infectious mononucleosis; and JASTRA, a cell line established from a normal male. All of the cell lines grow in suspension except for LICR-LON, which is partially adherent. Cell lines were tested regularly for mycoplasma, and were free from contamination [15].

The culture medium consisted of RPMI 1640 (Gibco, Paisley, U.K.) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, 0.5% $NaHCO_3$ and 1% phenol red. A stock solution of MTT (Sigma, St Louis, Missouri, U.S.A.) at 5 mg/ml was made with indicator-free phosphate-buffered saline (PBS; Oxoid, U.K.). The solution was filtered through a 0.2-μm Acrodisc filter (Gelman Sciences, U.S.A.) before use. The stock solution was stored in the dark at 4°C for a maximum duration of 3 weeks. It was diluted as required by the addition of filtered PBS.

MTT assay

All plates were established and harvested in an identical manner. Exponentially-growing cells were resuspended in fresh culture medium, and the cell densities adjusted as required for each experiment. Cells were plated in round-bottomed 96-well (12 × 8) microtitre plates (Bibby Sterilin, U.K.). The first column of eight wells in each plate was used as a background control and culture medium only was dispensed to these wells. Eight replicate wells (one column) containing cells in 200 μl of culture medium were plated for each data point. The plates were sealed with tape to minimise evaporation, and incubated at 37°C in an humidified incubator containing 5% CO_2 until the time of harvest.

Harvesting of plates

MTT solution (50 μl) was added to each well of the microtitre plate and the plates incubated for a further 3–4 h (the MTT concentrations and incubation times used are listed in the Results).

The plates were then centrifuged at 400 *g* to collect the formazan crystals at the bottom of each well. The medium and MTT solution were aspirated from the formazan crystals using a fine-bore Pasteur pipette and replaced with 200 μl of dimethylsulphoxide (DMSO; Fisons, U.K. reagent grade [11, 16]). The contents of each well were thoroughly mixed by gentle stirring and pipetting with a multi-pipette, and the optical density resulting from the solubilisation of the formazan crystals was read immediately using a multiwell spectrophotometer at 540 nm (Titertek Multiskan). The spectrophotometer was blanked with an empty plate before use. Optical densities greater than 1.999 absorbance units could not be read with this machine. The mean value of each column of replicate wells was calculated, and the value from the column of background control wells subtracted to obtain a "corrected" optical density for each data point.

Three types of experiment were performed.

Optimum MTT concentration and time

A fixed number of non-irradiated cells (1×10^4 per well for JASTRA and DEW1, and 2×10^4 per well for LICR-LON and K562) suspended in culture medium were plated into five columns of replicate wells. Each column was incubated with 50 μl of MTT solution at concentrations of 1, 2, 3, 4 and 5 mg/ml (final concentrations of MTT were 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml). In each experiment, four replicate plates were harvested at hourly intervals from 2 to 5 h after the addition of MTT.

Relationship between cell number and optical density

Non-irradiated cells were resuspended in fresh culture medium and eight replicate wells were plated with cell numbers ranging from 0 to 5×10^4 cells per well. The plates were harvested as described using the MTT concentrations and times from the previous experiments. A minimum of three independent experiments were performed with each cell line for the MTT time and concentration experiments and for the cell number and optical density experiments.

Radiation survival using the MTT assay

Preliminary experiments to test the growth patterns of cells plated at different densities and irradiated with different doses of ionising radiation were performed (data not shown). The cell densities and radiation doses used in the final experiments were: K562—1000, 1500, 2000 and 3000 cells per well, 0–8.0 Gy; LICR-LON—1500, 2000, 2500 and 3000 cells per well, 0–4.0 Gy; DEW1—2000, 3000, 4000 and 5000 cells per well, 0–2 Gy; JASTRA—2000, 3000, 4000 and 5000 cells per well, 0–2.0 Gy.

Cells were irradiated at a density of 1×10^6 cells per ml using a Stabilipan X-ray machine fitted with a Th II filter (Siemens, Germany) at 250 kV with a dose rate of 37 cGy per min at room temperature. They were resuspended in fresh culture medium and diluted as required. Seven to nine replicate plates were set up containing non-irradiated cells and irradiated cells from four radiation dose levels at two different cell densities. The plates were incubated and then the cells harvested at 24-h intervals after the completion of plating for 6–9 days. On day 5, 50 μl of culture medium were added to each well in all plates to minimise the effects of evaporation and to provide nutrition for the cells. Four independent experiments were performed with each cell line.

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Revised 7 Apr. 1994; accepted 27 Apr. 1994.

After harvest, the "corrected" optical density from each column of replicate wells was converted back to a cell number using the data from the experiments that defined the relationship between optical density and cell number. A daily graph of the cell numbers at each dose level was drawn for both cell densities over the duration of an experiment. Experiments in which the cells grew poorly, or the control cells became confluent too early in the experiment to allow extrapolation of the growth curve, were excluded from analysis. Four to six graphs with results from at least three independent experiments were used for each cell line. The surviving fractions from these graphs were calculated by dividing the number of irradiated cells by the number of non-irradiated cells for each radiation dose both at a time when the irradiated cells had achieved exponential growth [6], and when the non-irradiated population of cells had undergone four or more doublings from the original plating number [4]. Extrapolation of the growth curve for the non-irradiated cells was usually required so that the surviving fractions could be calculated when irradiated cells had regained their maximal growth rate [6].

Radiation survival using a clonogenic assay

Radiation survival was measured by colony growth in 0.5% agar. The method has been fully described previously [17]. Briefly, exponentially-growing cells were irradiated with doses of 0, 1.0, 2.0, 3.0 and 4.0 Gy (K562 received doses of 0, 2.0, 4.0, 6.0 and 8.0 Gy). The cells from each dose level were counted and diluted to twice the plating number for each cell line (data not shown) in 1 ml of 2 × RPMI containing 20% fetal calf serum and an additional 1% glutamine. The cell suspension was mixed with an equal volume of liquified, 1% agar (Difco Noble, U.S.A.) in double-distilled water and with August rat red blood cells [2] at a final concentration of 1:80 (v:v). For each dose level, four replicate wells were plated with 1 ml of the cell and agar suspension. The plates were incubated in a humidified incubator containing 5% CO_2 at 37°C. Culture medium (0.5 ml) was added to all wells after 4–5 days, and then as required to prevent dehydration of the plates. Colonies containing 50 or more cells were counted under the 10× objective of an inverted phase contrast microscope after 7–15 days. The surviving fractions were calculated by dividing the mean number of colonies from the wells containing irradiated cells by the mean number of colonies from the wells containing non-irradiated cells. All experiments were performed a minimum of three times.

RESULTS

Optimum MTT concentration and time

The results from LICR-LON and DEW1 are shown in Figure 1a and b. Increasing concentrations of MTT produced higher optical densities until a maximum was reached. The highest concentration of MTT resulted in a reduced optical density at all incubation times for both cell lines. An excess of MTT has previously been observed to result in a reduction in formazan production in other cell lines [18]. DEW1's results show little difference between the optical densities obtained after 3 and 4 h incubation, possibly due to MTT toxicity inhibiting cell metabolism during longer incubation periods.

The MTT concentrations and times selected for LICR-LON and DEW1 were 2 mg/ml for 4 h and 2 mg/ml for 3 h, respectively. The results from the experiments using K562 and JASTRA were similar to those from LICR-LON (data not shown) and the MTT concentrations and incubation times used for these cell lines were 3 mg/ml for 4 h and 2 mg/ml for 4 h.

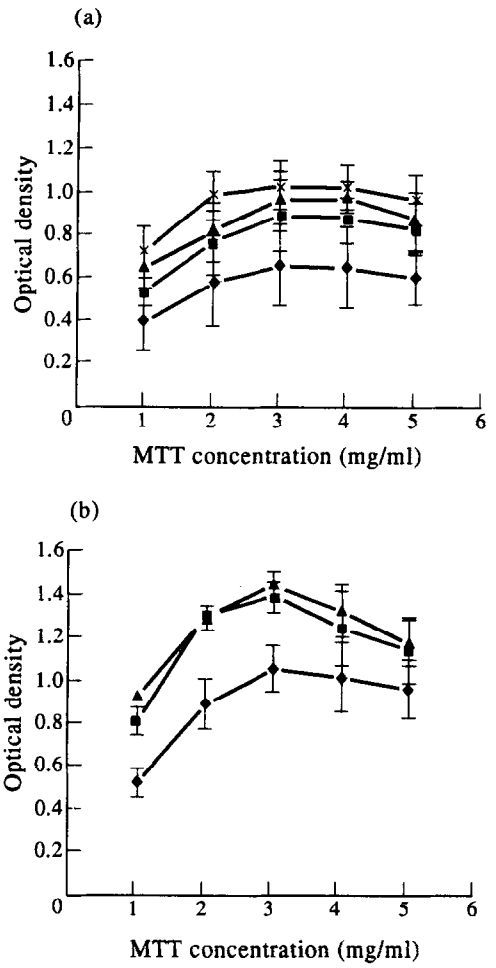

Figure 1. The relationship between optical density and MTT concentration and incubation time. Each point is the mean of three independent experiments. The standard deviations for each point are shown on the graph (a) LICR-LON: ◆ 2 h (0.18), ■ 3 h (0.13), ▲ 4 h (0.11), × 5 h (0.12). (b) DEW1: ◆ 2 h (0.10), ■ 3 h (0.10), ▲ 4 h (0.08).

Relationship between cell number and optical density

The results from these experiments are shown in Figure 2. A line of best fit was drawn by eye for each cell line. The relationship between cell number and optical density was not linear for our cell lines at higher cell numbers.

Radiation survival using the MTT and clonogenic assays

An example of a curve from the MTT assay for each cell line is shown in Figure 3a to d. The cell lines show different growth rates under the experimental conditions. The irradiated cells did not always achieve the same rate of exponential growth as the non-irradiated cells, for example, LICR-LON after treatment with 4 Gy. We did not always obtain exponential growth from K562 after irradiation with 6 and 8 Gy nor for LICR-LON at 4 Gy. We, therefore, did not analyse the surviving fractions from these dose levels.

We calculated the surviving fractions from the MTT data on several days when the irradiated cells had reached their maximum growth rates, and when the non-irradiated cells had undergone four or more doublings from the original plating number. The MTT surviving fractions are shown together with those measured by clonogenic assay in Figure 4a to d. The values for the surviving fractions after irradiation with 1.0 and 2.0 Gy

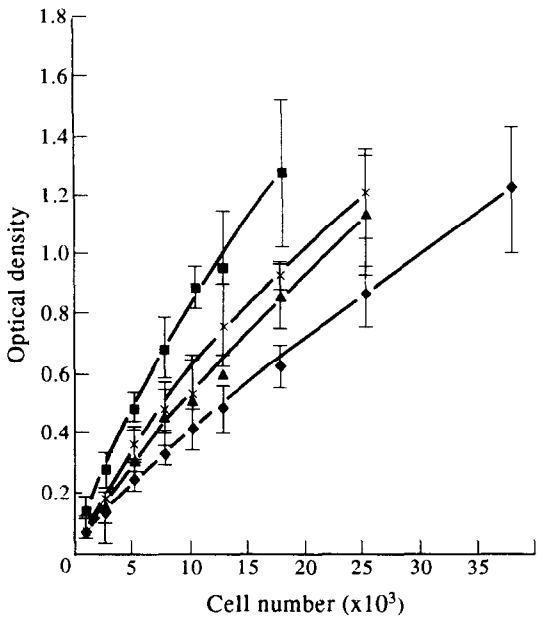

Figure 2. The relationship between optical density and cell number. Each point is the mean of three to four independent experiments. The standard deviation of each set of experiments is shown on the graph. ◆ K562, ■ LICR-LON, ▲ DEW1, × JASTRA.

are listed in Table 1. We were able to rank the cell lines in order of radiosensitivity using both methods for the analysis of MTT surviving fractions and without recourse to the clonogenic data. However, measuring the surviving fractions after the non-irradiated cell population had undergone four doublings often required extrapolation of the growth curves for non-irradiated and irradiated cells, and was thus more speculative.

Using the exponential growth method, the surviving fractions from all dose levels could be obtained from analysis on 1 day only in some cell lines (for example, DEW1, 0.5–2.0 Gy on day 7). However, the best correlation between the two assays was sometimes achieved when the surviving fractions from different dose levels were analysed on different days. This is illustrated in Figure 4b. The MTT surviving fractions for LICR-LON are closest to the clonogenic data on day 3 for 1.0 Gy, day 4 for 2.0 Gy and day 6/7 for 3.0 Gy. Such analysis could only be carried out retrospectively. The surviving fractions from the MTT assay showed a tendency to higher values than the clonogenic assay at the higher radiation dose levels, for example, JASTRA and DEW1 at 2.0 Gy.

DISCUSSION

In our experimental procedure, we chose to add the MTT solution directly to the medium and cells, rather than aspirate the medium from the cells prior to the addition of the MTT solution. In preliminary experiments, we found that aspiration of the medium without MTT from the non-adherent cells resulted in cell loss even after centrifugation of the plates. This cell loss was minimised by the addition of MTT, as aspiration of the medium and cells from the purple formazan crystals, which could be more clearly seen, was considerably easier. It must also be mentioned that we did not perform any experiments to examine the effects of August rat red blood cells on cell growth in the MTT assay. Any possible effect that these cells may have had on the MTT survival curves is not known.

Use of the MTT assay to measure the number of viable cells depends on the assumption that the production of formazan crystals and the resultant optical density is proportional to the number of cells. The relationship between cell number and optical density is not always linear and must be investigated for each cell line [6, 10, 19]. Our cell lines also showed considerable variation in the non-linear relationships between cell number and optical density (Figure 2). We, therefore, chose to reconvert the optical densities back to cell numbers for the analysis of surviving fractions and to allow extrapolation of the growth curves.

We also found it useful to determine the optimum MTT concentration and incubation time for each cell line. The results (Figure 1a, b) show that reduced formazan production is possible with high MTT concentrations [18], and that the optimal incubation time may vary between cell lines.

Preliminary experiments were necessary to establish the best numbers for use with each cell line. K562 grew well when plated at 1000 cells per well, whereas JASTRA and DEW1 did not grow reliably at densities below 2000 cells per well (data not shown). It was sometimes difficult to find a balance between plating the number of cells per well required for reliable growth and plating higher numbers of cells that could result in the non-irradiated cells (or those exposed to a low dose level of radiation) achieving confluence. Confluence can result in overestimation of the surviving fractions [6, 7, 20].

In some experiments, the irradiated cells showed an initial increase in number at a rate compatible with exponential growth before either slowing in growth or declining in number (for example, JASTRA at 2 Gy, Figure 3d). This pattern is consistent with lethally-irradiated cells maintaining normal metabolic function before undergoing mitotic death [21]. It can, therefore, be misleading to measure the cell surviving fraction before delayed reproductive cell death has occurred [4, 10].

The cells exposed to the higher dose levels of radiation did not always achieve the same rate of growth as the non-irradiated cells, for example, LICR-LON exposed to 3 and 4 Gy. Although increasing the duration of our experiments may have provided time for these cells to attain exponential growth, we chose not to continue our experiments for longer than 9 days. This was due to the practical limitation of plating sufficient replicate plates for daily harvest over a prolonged period. However, an increased number of dead cells from the high doses of radiation may also have inhibited the growth rate or the mitochondrial function of the remaining live cells. The inability to obtain regrowth in these cell populations restricted the dose range for which we could use the MTT assay.

The best correlation between the MTT and clonogenic data was obtained for the cell lines DEW1 and JASTRA (Table 1). In these cell lines, the surviving fractions measured after the non-irradiated cells had undergone four or more doublings were similar to those measured when the irradiated cells were in exponential growth. However, with K562 and LICR-LON, the best correlation between the MTT and clonogenic data was found retrospectively by taking the surviving fraction from different days for the exponentially-growing irradiated cells. Analysis on any 1 day over- or underestimated the surviving fraction compared to the clonogenic assay for at least one radiation dose level (Figure 4a and b). In these cell lines, measurement of the surviving fraction after four or more doublings was sufficient to rank the cell lines in order of radiosensitivity, although there was still relatively poor agreement between the MTT and clonogenic results for LICR-LON at 2.0 Gy. The need to use surviving fractions from different days for different

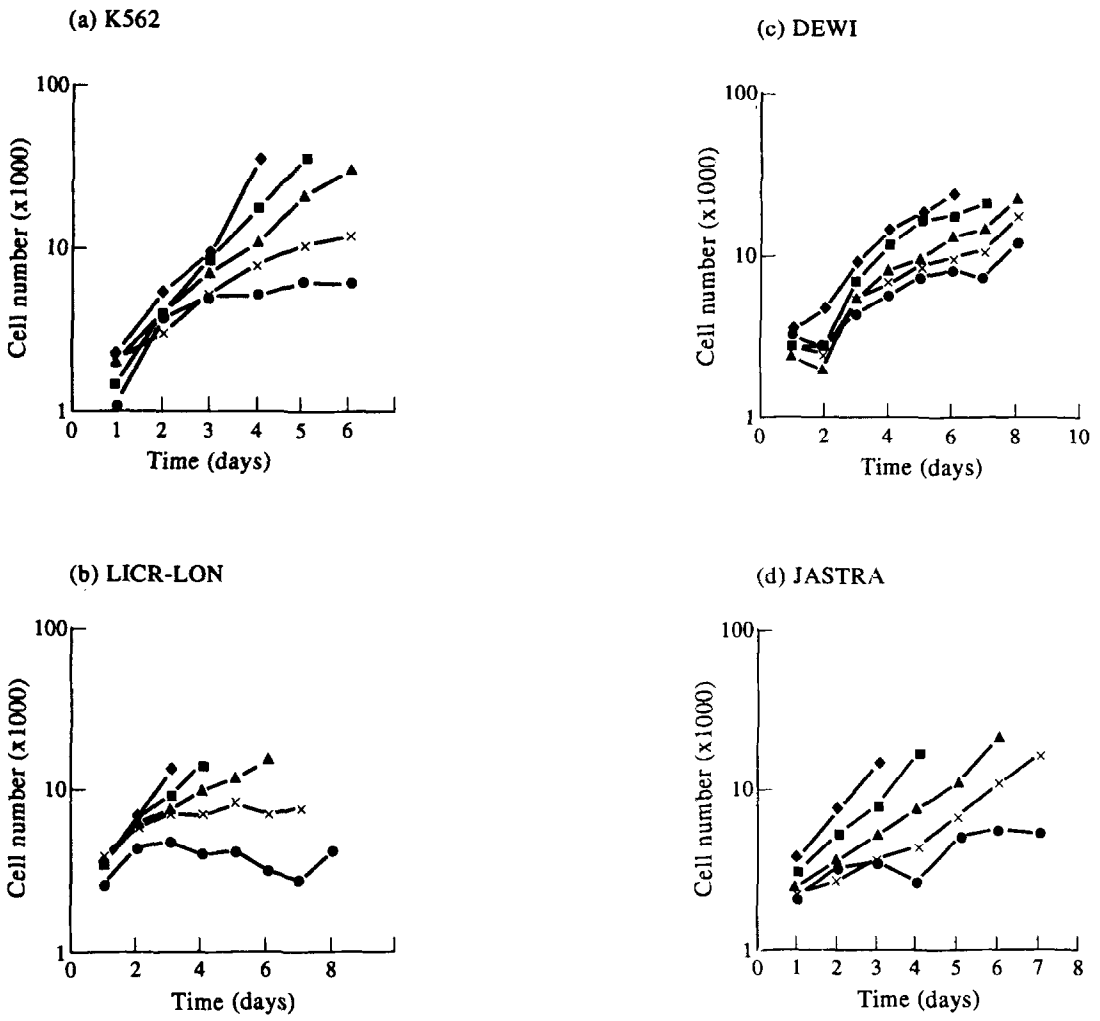

Figure 3. Growth curves for non-irradiated and irradiated cells. Each point was obtained by converting the mean optical density of eight replicate wells (after subtraction of the mean optical density for the background control wells) to a cell number using the information in Figure 2. (a) K562 at 1000 cells per well. ◆ 0 Gy, ■ 2.0 Gy, ▲ 4.0 Gy, × 6.0 Gy, ● 8.0 Gy. (b) LICR-LON at 1000 cells per well. ◆ 0 Gy, ■ 1.0 Gy, ▲ 2.0 Gy, × 3.0 Gy, ● 4.0 Gy. (c) DEW1 at 3000 cells per well. ◆ 0 Gy, ■ 0.5 Gy, ▲ 1.0 Gy, × 1.5 Gy, ● 2.0 Gy. (d) JASTRA at 5000 cells per well. ◆ 0 Gy, ■ 0.5 Gy, ▲ 1.0 Gy, × 1.5 Gy, ● 2.0 Gy.

dose levels may reflect the greater range of radiation doses used in these cell lines.

The surviving fractions measured by the MTT assay were higher than those from the clonogenic assay in all of our experiments, except for LICR-LON, when measured after four or more doublings. This may be due to persistent mitochondrial function in lethally-irradiated cells or cells incapable of division [5]. Alternatively, the surviving cells may have overgrown the dead or damaged cells to produce a higher optical density. The MTT assay was also found to underestimate the anti-proliferative efficacy of several anti-neoplastic drugs when compared to surviving fractions obtained by cell counting [22]. It must be remembered that the MTT assay measures the surviving fraction of a population of clonogenic and non-clonogenic cells, whereas the colony-forming assay measures the surviving fraction of clonogenic cells only.

Finally, we did not find the MTT assay difficult to perform in suspension cell lines, although large clumps of cells were occasionally difficult to solubilise. The use of fine-bore Pasteur pipettes and round-bottomed wells facilitated the aspiration of medium and MTT without disruption of the formazan crystals. However, residual medium is known to affect the optical density of the solution resulting from the addition of organic solvent [16, 18], and, although we were usually able to remove most of the medium, variation in the amount remaining may have contributed to the range of optical densities observed for replicate wells (data not shown). Although less time is clearly needed to perform one MTT experiment compared to a clonogenic assay, the total duration of the MTT experiments was increased by the need to collect preliminary data, such as the relationship between cell number and optical density for each cell line, before the measurement of radiation survival.

In conclusion, we found that we could use the MTT assay to measure the relative radiosensitivity of suspension cell lines. The survival fractions could be measured either after the irradiated cells had regained exponential growth, or when the non-irradiated cells had undergone four doublings from the original plating number. However, the best correlations between the results from MTT and clonogenic assays for K562 and LICR-LON required analysis on different days for different radiation dose levels. This fact, together with the limited radiation dose range for which satisfactory regrowth could be obtained in our cell lines, may restrict the use of the MTT assay for the measurement of radiation survival.

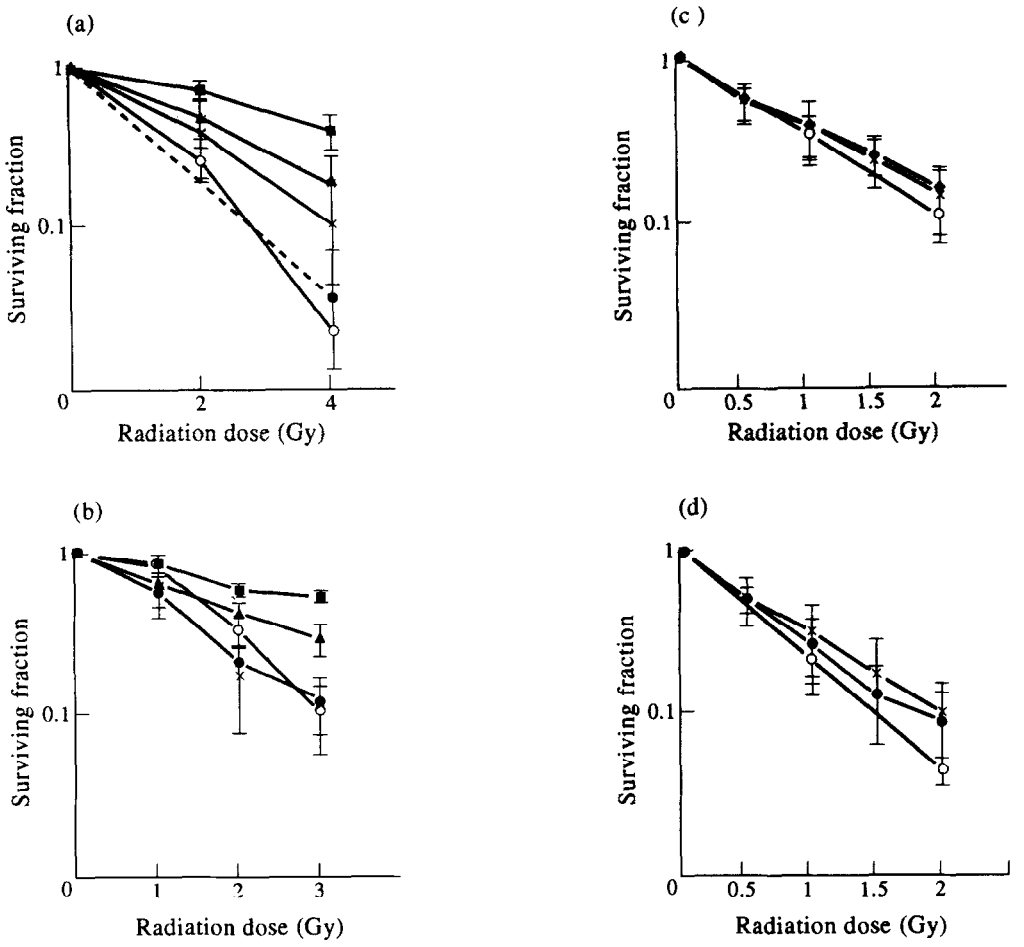

Figure 4. Comparison of surviving fractions measured by MTT and clonogenic assays. Each data point from the MTT assay is the mean of four experiments for K562 and LICR-LON, five experiments for JASTRA and six experiments for DEW1. Each point from the clonogenic assay is the mean of three experiments. The pooled standard deviation for each group of experiments is given in the brackets. (a) K562: 2.0–4.0 Gy, ○ clonogenic assay (0.05), ■ MTT assay day 3 (0.07), ▲ MTT assay day 4 (0.12), ● MTT assay day 6 (0.08), × MTT four doubling times (0.12). (b) LICR-LON: 1.0–3.0 Gy, ○ clonogenic assay (0.07), ■ MTT assay day 3 (0.09), ▲ MTT assay day 4 (0.08), ● MTT assay day 6/7 (0.11), × MTT four doubling times (0.11). (c) DEW1: 0.5–2.0 Gy, ○ clonogenic assay (0.06), ◆ MTT assay day 7 (0.12), × MTT four doubling times (0.13). (d) JASTRA: 0.5–2.0 Gy, ○ clonogenic assay (0.06), ● MTT assay day 6 (0.18), × MTT four doubling times (0.14).

Table 1. Surviving fractions from MTT and clonogenic assays

Cell line	Plating efficiency (%)	Radiation dose (Gy)	Surviving fraction MTT assay. Exponential growth (S.D.)	Surviving fraction MTT assay. Four or more doublings (S.D.)	Surviving fraction. Clonogenic assay (S.D.)
LICR-LON		1.0	0.87 (day3) (0.12)	0.63 (0.17)	0.83 (0.09)
DEW1		1.0	0.41 (day7) (0.16)	0.40 (0.17)	0.36 (0.10)
JASTRA		1.0	0.28 (day6) (0.12)	0.34 (0.16)	0.23 (0.09)
K562	5.04 (2.96–9.04)	2.0	0.64 (day5) (0.18)	0.71 (0.13)	0.53 (0.08)
LICR-LON	0.86 (0.78–1.02)	2.0	0.43 (day4) (0.10)	0.18 (0.05)	0.35 (0.07)
DEW1	1.85 (1.76–1.96)	2.0	0.17 (day 7) (0.05)	0.16 (0.07)	0.12 (0.04)
JASTRA	1.67 (1.42–2.07)	2.0	0.10 (day6) (0.05)	0.11 (0.06)	0.05 (0.01)

Each point from the MTT assay is the mean of four experiments for K562 and LICR-LON, five experiments for JASTRA and six experiments for DEW1. Each point from the clonogenic assay is the mean of three independent experiments. Each plating efficiency is the mean from three independent experiments, with the range shown in parentheses. The pooled standard deviation for each group of experiments is given in parentheses.

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Acknowledgements—The authors are grateful to Dr Elizabeth Foster for her help in obtaining the August rat blood, to Dr Simon Langdon for his advice on the clonogenic assay and to Dr Andrew Carothers for his help with the statistical analysis. A. Slavotinek was funded by the National Radiological Protection Board while pursuing this work. The cell lines K562, LICR-LON, DEWI and JASTRA were kindly provided by the MRC Human Genetics Unit.