Use of the Tetrazolium Assay in Measuring the Response of Human Tumor Cells to Ionizing Radiation

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ABSTRACT

Three human tumor cell lines of widely differing radiosensitivity were used to examine the characteristics of the 3-[4,5-dimethyl(thiazol-2-yl)-3,5-diphery]tetradium bromide (MTT) assay and to select suitable conditions for its use in assessing the response of cells to ionizing radiation. The optimal concentration of MTT and the time of incubation of the cells with MTT were individualized for each cell line. The relationship between absorbance and cell number was not linear over the wide range of cell numbers that were used. A calibration curve of absorbance against cell number for each cell line was therefore used.

Using the assay to quantify metabolically viable cells, growth curves of irradiated and unirradiated cells were constructed on days 0–14 after irradiation. Accurate surviving fractions could be calculated only when cells were in exponential growth. Using this modification to its interpretation, the MTT assay was able to provide a reproducible measure of survival, which compared well with clonogenic cell survival measurements. However, the necessity to optimize conditions of the MTT assay for each cell line severely limits its usefulness in determining the radiosensitivity of cells in primary human tumor cultures.

INTRODUCTION

The MTT[3] assay is a novel method of quantifying metabolically viable cells through their ability to reduce a soluble yellow tetrazolium salt to blue-purple formazan crystals (1). The crystals are thought to be produced by the mitochondrial enzyme succinate dehydrogenase (2) and can be dissolved and quantified by measuring the absorbance of the resultant solution. The absorbance of the solution is related to the number of live cells. By using 96-well microtiter plates and a multiwell spectrophotometer (enzyme-linked immunosorbent assay plate reader) this assay can be semiautomated to process a large number of samples and provide a rapid objective measurement of cell number. A number of laboratories are using this assay and various modifications have been introduced (3–7).

The MTT assay was first used to study the *in vitro* effects of lymphokines (1, 3, 8, 9). It was then developed to measure chemosensitivity in human tumor cell lines (4, 6, 7, 10) and more recently fresh human leukemia cells (11, 12). Its widest application has been, however, in the new disease-oriented drug screening program at the National Cancer Institute (5).

The use of the MTT assay in assessing the response of cells to ionizing radiation has been less widely studied (13, 14). Traditionally radiation cell survival is measured using a clonogenic assay and this remains the established method of choice. However, there are situations where it is not satisfactory, *e.g.*, assay of non-colony forming cells and rapid assessment of cell survival. This study has examined the use of the MTT assay as an alternative to the clonogenic assay and has assessed its value

in measuring the response of cultures of primary human tumors to ionizing radiation.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. Three human tumor cell lines have been used. HX142 was derived from a neuroblastoma by Dr. J. M. Deacon in this Department. MGHU1 and RT112 were both originally derived from transitional cell carcinomas of the bladder (15, 16). HX142 is highly radiosensitive while MGHU1 and RT112 are radioresistant (17). All three cell lines grew as monolayers in Ham's F-12 medium containing penicillin and streptomycin. Medium for HX142 and RT112 were supplemented with 10% fetal calf serum while for MGHU1 aseptic calf serum was used at a concentration of 20%. All cells were maintained at 37°C in a humidified atmosphere of 90% N_2, 5% CO_2, and 5% O_2.

All cells were regularly assessed for freedom from *Mycoplasma* contamination.

MTT Solution. MTT was dissolved in sterile phosphate-buffered saline at 5 mg/ml and stored for no more than 3 weeks in the dark at 4°C. After final dilution with prewarmed sterile unsupplemented culture medium, the solution was filtered through a 0.22-μm filter to remove formazan crystals.

MTT Assay. Cells were harvested from exponential-phase maintenance cultures using trypsin:Versene (0.05:0.02%) treatment of monolayer cultures. Single-cell suspensions were prepared, cells counted using a hemocytometer and then dispersed within replicate 96-well microtiter plates to a total volume of 200 μl/well. Eight duplicate wells were used for each determination.

Plates were maintained at 37°C in a humidified atmosphere of 90% N_2-5% CO_2-5% O_2. A 24-h preincubation time was allowed prior to irradiation.

To perform the MTT assay, culture medium was removed from the wells ensuring that the monolayer of cells was not disturbed. MTT solution (100 μl) at appropriate concentrations was then added to each well and the plates incubated at 37°C for 3–5 h, depending upon individual cell line requirements (see below). Following incubation, cells were inspected using low power microscopy to confirm reduction of the tetrazolium and to assess confluency of the monolayer. The remaining MTT solution was then removed and 150 μl of DMSO was then added to each well to dissolve the formazan crystals. Plates were shaken for 5 min on a plate shaker to ensure adequate solubilization.

Absorbance readings on each well were performed at 540 nm (single wavelength) using a Titertek Maltestian MCC plate reader. A reference wavelength was not used inasmuch as this made little difference to the absorbance readings obtained. Control wells for absorbance readings contained no cells or medium but MTT solution was added as per experimental wells, and removed after incubation, and DMSO was then added.

All experiments were performed at least twice.

Irradiation Procedures. All irradiations were performed using a ^{60}Co source in a 2000-Ci telecobalt irradiation room. The irradiation dose rate was 150 cGy/min, as assessed by an Ionex type 2500/3 dosemeter.

Concentration and Time of Incubation with MTT. The concentration and time of incubation with MTT solution used in this assay are known to affect the absorbance measurements obtained from cell lines (3, 6). For instance our 3 cell lines when incubated with 1 mg/ml MTT for 4 h demonstrated a range of absorbance measurements for the same cell number. To select conditions for each cell line, unirradiated cells at serial concentrations, from 312 to 10,000 cells/well were incubated with a range of concentrations of MTT (0.125–5 mg/ml) for 3–4 h and

with 1 mg/ml of MTT for a range of times (1-7 h). Concentrations of MTT that afforded the largest range of absorbance with varying cell numbers and the time that showed least change in absorbance with variations of up to 30 min were chosen: 0.25 mg/ml for 2.5 h for HX142; 1 mg/ml for 5 h for MGHU1; and 0.5 mg/ml for 3 h for RT112. A standard concentration and time of incubation with MTT could have been used for each cell line as long as the relationship between cell number and absorbance was determined (see below), but individualizing conditions was thought to increase the sensitivity and accuracy of measurements.

RESULTS

Relationship between Absorbance and Cell Number

For each cell line, cells were dispensed into 96-well plates in serial dilutions from 400,000 to 156 cells/well and incubated for 24 h at 37°C to allow attachment. The intensity of MTT conversion was then assessed at 24 h using the chosen conditions for each cell line (see "Materials and Methods"). In duplicate wells cells were removed by trypsin-Versene treatment and counted using Lissamine green dye exclusion in order to confirm cell concentration per well. Fig. 1 shows the relationship between absorbance and cell number in representative experiments for each cell line. Readings were highly reproducible and usually differed by less than 0.04 absorbance unit for the same MTT conditions. The relationship between absorbance and cell number is far from linear. Such curves were therefore used to convert absorbance measurements into equivalent cell numbers for each cell line.

Time Course of Growth of Treated and Control Cultures

For each cell line cells were dispensed into 96-well plates using 6 serial concentrations between 5000 and 156 cells/well. Plates were irradiated 24 h later with up to 6 dose levels ranging from 1 to 20 Gy. Cells were subsequently incubated at 37°C. Medium was changed every 7 days. The MTT assay was performed at intervals for up to 14 days following irradiation and by means of the calibration curves estimates were obtained of the number of cells per well.

Fig. 2 shows representative experiments relating the estimated cell number per well and time after irradiation. For the first 4-6 days the untreated cells approached exponential growth but when the estimated cell number exceeded 4-10 × 10^4 (depending on the cell line) the growth rate declined. Saturation density was quickly reached and the subsequent
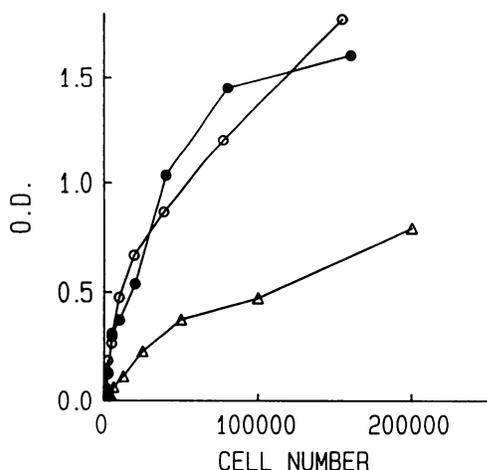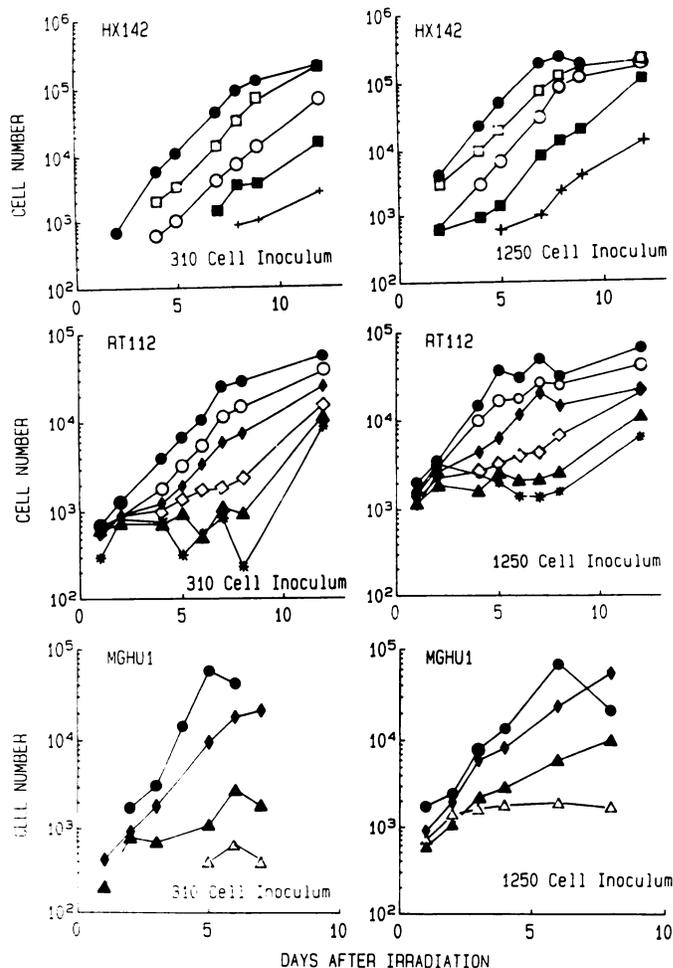

Fig. 2. Growth curves for the three cell lines following irradiation. Untreated controls (●), 1 Gy (□), 2 Gy (○), 3 Gy (■), 4 Gy (+), 5 Gy (◆), 7.5 Gy (◇), 10 Gy (▲), 12.5 Gy (*), and 15 Gy (△). Initial cell inoculum was 310 and 1250 cells/well.

decline reflected loss of cells from the confluent monolayer. Irradiation suppressed growth in a dose-dependent manner. In most cases there was a lag period after irradiation followed by regrowth at approximately the same growth rate as the unirradiated controls. However, in some cases (especially at high radiation dose levels) the rate of regrowth was less than that of controls.

Derivation of Cell Survival Curves

Fig. 2 demonstrates that simply comparing cell numbers at any fixed time after irradiation will not produce meaningful surviving fractions. This is due both to the dose-dependent lag period after irradiation before regrowth is obtained and to the time taken by control cultures to reach confluency. The end point in a growth assay when adopted to measure cell survival is the ability of the total cell population to regain the growth rate of the control population, whereas in a clonogenic assay the regenerative potential of a small proportion of clonogenic cells is being measured. Thus in growth assays, surviving fractions are obtained when treated cultures attain exponential regrowth at the control growth rate. Using this definition of survival, two approaches can be used to derive cell survival curves.

Vertical Displacement of Growth Curves. When the treated cultures regrew at the same rate as controls it was a simple matter to evaluate from the vertical displacement of the curves

Fig. 1. Calibration curve for absorbance against cell number for HX142 (△), RT112 (●), and MGHU1 (○).

1393

an estimate of the level of cell kill (Fig. 3, X). When this was not the case or when the treated cultures did not attain exponential regrowth during the period when controls were growing exponentially, exponential extrapolation at the control growth rate was used to obtain an estimate of cell kill (Fig. 3, Y). This latter method uses a predicted control cell number, and in Fig. 4 such results are shown as solid circles.

Fig. 4 shows surviving fraction, as a function of radiation dose, calculated from the ratio of estimated cell numbers when the control and/or treated cells were growing exponentially at the same growth rate. The multiple points at each dose level come from repeat experiments using a range of seeded cell numbers. The survival curves produced by averaging in this way were quite reproducible.

Method of Graded Inocula. An alternative approach is the determination of growth as a function of size of inoculum. Results obtained in this way are shown in Fig. 5. A sighting experiment to define the time course of growth of treated and control cultures is necessary in order to choose the optimum time at which to carry out the measurements, i.e., when the majority of regrowth curves show active regrowth and as far as possible are parallel. Curves of estimated cell number against inoculum size (Fig. 5) usually show a roughly linear initial region, followed by a tendency to saturate, and represented the growth from the different cell inocula on that day. The slope of this initial region was found to provide a good measure of surviving cell number and the ratio of treated slope to control slope gives a reliable indication of surviving fraction.
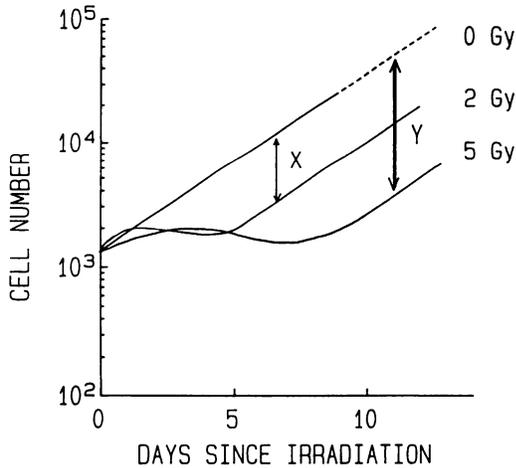

Fig. 3. Extrapolation of MTT growth curves. Curves for 0, 2, and 5 Gy based on data from RT112. The displacement of the curves for the treated groups (X, 2 Gy; Y, 5 Gy) were taken when cells were in exponential growth. - - -, exponential extrapolation of growth curve.

Comparison of MTT Assessment of Cell Survival and Clonogenic Assay

Fig. 6 shows a comparison between the results obtained by the two methods described above and the clonogenic assay routinely performed in this Department (17). In each case the data have been fitted by the linear quadratic equation

$$\ln SF = -\alpha D - \beta D^2$$

Incorrect Measurement of Survival Fractions

Simply comparing cell numbers at any fixed time after irradiation does not yield a reliable result. Fig. 7 shows the "apparent" survival curves obtained in this way. If survival is measured during the lag phase, i.e., too early, or when control cultures have reached confluency, i.e., too late, survival is overestimated. Also, a single day may not be sufficient to obtain survival fractions for the full range of doses. This is best illustrated in Fig. 2 for RT112 (312 cell inoculum). Growth of treated RT112 cells becomes exponential on day 5 at low doses, but much later for higher doses. This explains why only the early part of the 5-day apparent cell survival curve approximates the true survival curve; the survival at higher doses would have to be derived at a later day, and if by then the control cultures had become confluent, forward extrapolation of control curves (as above) would be required.

DISCUSSION

Radiation cell survival is usually measured using clonogenic assays and this is probably the most reliable method. Limitations, however, include the time taken for colonies to form and the inability to measure survival in cells which do not grow as colonies. The search for alternative assays which may be more appropriate under certain circumstances has been the subject of much research over the years.

Growth assays are an alternative method of measuring cellular response to injury. These assays rely on quantifying growth of cells in short term culture. Various methods of measuring the number of living cells have been used, e.g., dye exclusion (18), isotope uptake (19), staining with crystal violet, and quantifying with computerized image analysis (20), and more recently staining with a fluorescent DNA-specific dye, Hoechst 33258 (21). The MTT assay quantifies metabolically viable cells by their ability to reduce MTT.

The advantages of the MTT assay include rapid semiautomated reading, objective assessment, comparative low cost, high reproducibility, low number of cells required, and the facility to quantify cells grown in suspension (22), on monolayer or in spheroids or colonies. However, there are 2 specific problems with the MTT assay: (a) as previously reported by other groups, we have confirmed that the absorbance produced by a particular

Fig. 4. Surviving fractions for the three cell lines treated with graded doses of irradiation as assessed using vertical displacement of growth curves. Individual points represent data from a minimum of two experiments, each with a range of seeded cell numbers. ●, where forward extrapolation of control curves was required.
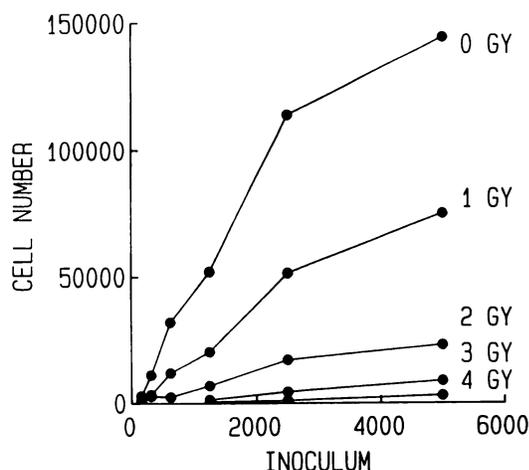

1394
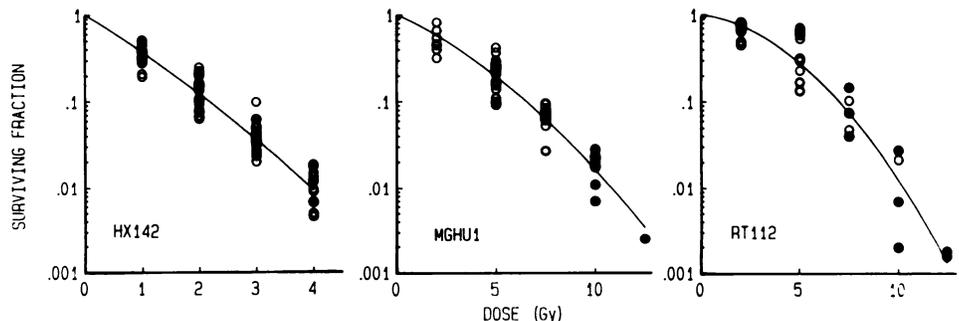
Fig. 5. Method of graded inocula. Data are given for HX142 assayed 5 days following irradiation. ●, cell number achieved by given inocula after different treatments.
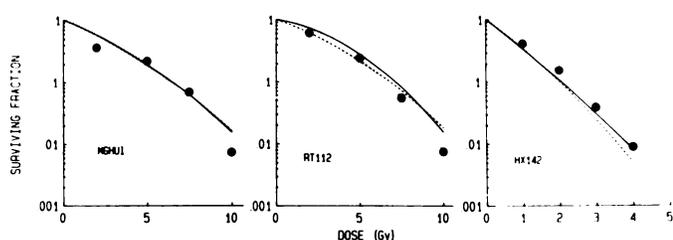

Fig. 6. Comparison of extrapolation method (——, curves from Fig. 4), method of graded inocula (●···●) and clonogenic survival curves (– – – –). ● on the graded inocula curves represent the mean surviving fraction of two experiments.
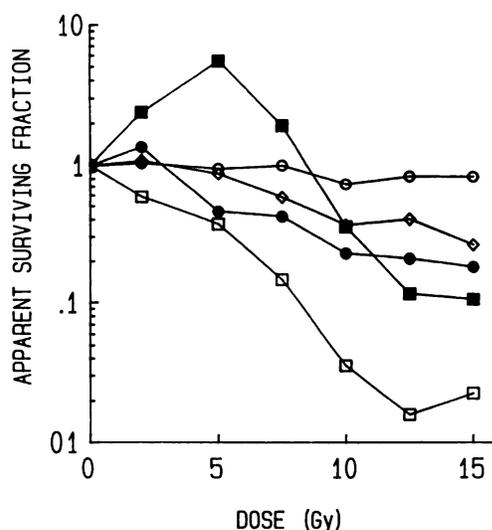

Fig. 7. Apparent surviving fractions measured on different days after irradiation. Data given for MGHU1 following 2500 cells/well inoculum. *Points* calculated by the ratio of treated over control cell numbers on Day 1 (O), Day 2 (◇), Day 3 (●), Days 5 (□), and Day 6 (■).

cell number can be greatly influenced both by the concentration of MTT used and by the time of incubation with MTT; (*b*) the relationship between cell number and absorbance over a wide range of cell numbers is not linear. Surviving fraction cannot be calculated by comparing absorbance as has been reported previously (13) except when low cell numbers are used that fall on the more linear part of the calibration curve (Fig. 1). Twentyman and Luscombe (6) have demonstrated a linear relation-

ship between absorbance and MTT/formazan solution in DMSO; thus possible explanations of this phenomenon include insufficient substrate (MTT) to saturate the enzymatic reaction, or inhibition of mitochondrial function, at higher cell numbers.

Growth assays in general carry the complication of the dose-dependent lag period after irradiation before regrowth is obtained (Fig. 2). This delay is probably due to the timing of cell death following irradiation (23, 24). This is important if an absolute measure, rather than a relative measure of survival is required.

Derivation of cell survival curves from growth curves has previously been performed by back extrapolation of growth curves (25, 26) and this method has been shown to compare well with clonogenic assays (26). However, the two approaches adopted here are thought to be more accurate because back extrapolation is sensitive to small changes in growth rate. The method of vertical displacement of growth curves gave results closest to the clonogenic assay. Multiple points at each dose level (Fig. 4) can be obtained from actual cell numbers at various time points along the exponential part of the growth curve, and growth curves from a range of seeded cell numbers can be used. The range of values at each time point is similar to the range obtained in a clonogenic assay and may be due to variation in growth in culture of different cell inocula as well as experimental error. The method of graded inocula is slightly less reliable, probably because only one point at each dose level is obtained, although this point dose incorporates a number of points relating cell number to cell inoculum. This latter method is also not always suitable; deriving a surviving fraction at a full range of doses is not always possible at 1 day because the full range of growth curves may not all become exponential on the same day (*e.g.*, Fig. 2, RT112), and as can be predicted from the examples in Fig. 2, this method will be totally unreliable if done blind, *i.e.*, unless the time course of treated and control cultures has already been determined and the day when growth curves are parallel is known.

The results obtained with the MTT assay are critically dependent on the conditions under which this assay is performed, the relationship between absorbance and cell number, and the way in which the results are interpreted. This is similar to other nonclonogenic assays, *e.g.*, the micronucleus assay (27), where conditions need to be carefully characterized for different cell lines before reliable results can be obtained. Once this is done, however, the MTT assay can provide a reproducible measure of survival which compared well with clonogenic cell survival measurements. Of note is the fact that the methods described for derivation of radiation cell survival curves from MTT growth curves are applicable to all growth assays.

The use of the MTT assay in measuring *in vitro* radiosensitivity of human tumors in short term culture is possible. However, it is necessary to individualize the relationship between absorbance and cell number for each tumor, which may be limiting if tumor cell yield from a biopsy is low. More importantly, the requirement for treated cell cultures to regain the exponential growth rate of the control culture before surviving fraction can be estimated will restrict the use of this or any other growth assay, until a reliable short term culture system for human tumors is available.

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1395

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