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Development of an *in Vitro* Assay for the Survival of Cells Suspended from BA1112 Rat Sarcomas*

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Abstract—*A method has been developed for the* in vitro *assay of the survival of BA1112 rat sarcoma cells following treatment* in vivo. *The BA1112 tumor was not adapted for growth in culture and was maintained by serial transplantation* in vivo. *The assay used a feeder layer of heavily irradiated cells to eliminate any changes in plating efficiency caused by the large number of dying cells which must be plated when assaying cell survival after intensive treatments. The plating efficiency of the tumor cells was not affected significantly by the age of the feeder layer (between* 1 *and* 4 days), *the density at which the experimental cells were plated or the interval between preparation of the cell suspension and plating (up to* 4 hr). *Using the* in vitro *assay a survival curve was determined for cells from tumors irradiated in air-breathing animals. This curve was similar to that determined previously for BA1112 tumor cells using an* in vivo *assay to measure cell survival.*

INTRODUCTION

ASSAYS which measure the response of tumors to therapy can be classified into two major types: those which measure the growth or control of tumors remaining *in situ* after treatment and those which measure the survival (viability) of cells suspended from tumors after treatment. Each approach has both advantages and limitations. The *in situ* endpoints are not applicable to all experimental studies. Tumor control experiments are limited to studying very intensive treatments and therefore measure the response of only the most resistant tumor cells; moreover, the results may be affected by the response of the normal tissues of the host. Tumor growth experiments measure only the gross changes in the total tumor volume, including changes in both the malignant cells and the stromal elements within the tumor. The results of tumor growth experiments may be affected not only by survival of the tumor cells, but also by such factors as changes in tumor cell proliferation patterns and changes in the number and proliferation patterns of the stromal cells within the tumor. Because of the limitations of the *in situ* methods, assays which measure the survival of individual tumor cells are also valuable for studies of tumor biology and tumor therapy. Methods such as the lung colony and TD_{50} assays, which measure tumor cell survival by testing the ability of the cells to form tumors in recipient animals, are expensive and time consuming and are inherently limited in accuracy [1, 2]. *In vitro* assays for cell survival, when available, offer a more rapid, precise and less expensive alternative for measuring cell viability. This paper reports the development of an *in vitro* assay for the survival of cells suspended from BA1112 tumors.

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MATERIALS AND METHODS

Tumor system

The BA1112 rhabdomyosarcoma arose in the mandible of an irradiated WAG/Rij rat in 1962 in the Netherlands [3] and has been maintained by serial transplantation in WAG/Rij rats. The current histology is that of a poorly differentiated rhabdomyosarcoma. It has not been possible to demonstrate a host immune response to the tumor [3; Moulder, personal communication]. The tumor was grown by subcutaneous inoculation of $2–3 \times 10^3$ cells between the ears of 6- to 12-week-old WAG/Rij rats of either sex as described previously [4]. Tumors inoculated in this manner had latent periods of 15–20 days and volume doubling times of 2–3 days at the size used for

these studies. Tumors were used for experiments at an average diameter of 10–15 mm. No differences in either plating efficiency or surviving fraction after irradiation were observed for tumors ranging from 5 to 20 mm in diameter. Tumors and host animals were screened yearly for viral contamination by the Section of Comparative Medicine at Yale according to published procedures [5]. Tumor cell suspensions were screened periodically for mycoplasma contamination by direct agar culture [6].

Suspension procedure

Single-cell suspensions were prepared from the tumors using a modification of the technique described by Reinhold [7]. Tumors were removed aseptically and chopped with a razor blade to produce a fine mash. The mash was filtered to remove blood and washed with 0.05% trypsin in Hanks' balanced salt solution (Gibco). The mash was then stirred with a magnetic stirrer in 100 ml of trypsin solution, which was initially at 37°C but was allowed to cool to room temperature during trypsinization. After 15 min the trypsin was inhibited with 5 ml of fetal bovine serum and the suspension was filtered to remove intact tumor fragments. The filtrate was centrifuged at 400 *g* for 5 min and the pellet was resuspended in 10 ml of tissue culture medium. The cells were counted with a hemacytometer, using trypan blue to identify cells with damaged membranes. During counting the suspension was examined to verify that a single-cell suspension had been obtained.

Feeder layer preparation

Feeder layers were prepared by a modification of the method of Puck and Marcus [8]. A tumor cell suspension was prepared, placed in a test tube and irradiated with 10,000 rad of X-rays as described previously [9]. The radiation dose was sufficient to eliminate colony formation by cells in the feeder layer. Cells were plated in 60×15-mm Petri dishes at a density of 1×10^6 dye-excluding cells/dish in a total volume of 6 ml of medium. Feeder layers were incubated at 37°C as described below. Feeder layers were generally used the day after preparation; older feeder layers were used in a few cases.

Assay procedure

A suspension prepared from an experimental or control tumor was diluted so that 1 ml of the suspension contained a cell number expected to produce approximately 100 colonies per dish. For treated tumors viability could not be predicted accurately; therefore, 3 or more dilutions were assayed to ensure that dishes with countable numbers of colonies were obtained. The cells, in 1 ml of medium, were added to dishes which contained feeder layers in 6 ml of medium. The medium used was Dulbecco's modified Eagle's medium with 1000 mg/l glucose supplemented with 16% fetal bovine serum, 0.1 mM non-essential amino acids, 1.1 mg/l sodium pyruvate and 1000 units/l each of penicillin and streptomycin. All ingredients were purchased from Gibco. The dishes were incubated at 37°C in a humidified atmosphere of 95% air/5% CO_2. After 14 days the colonies were rinsed with saline and then simultaneously fixed and stained with formaldehyde and crystal violet in methanol. For counting the dishes were projected on a screen at a magnification great enough so that individual cells could be visualized. Colonies which contained more than 50 cells were counted. Plating efficiencies were calculated as the mean number of colonies formed per 100 dye-excluding cells plated in 5 replicate dishes. Surviving fractions were calculated as the ratio of the plating efficiencies of the treated tumor cells to the plating efficiencies of untreated tumor cells which had been plated on the same day.

Irradiation procedure

Unanesthetized rats confined in Plexiglass boxes were irradiated with 250 KeV, 15 mA X-rays with 2 mm Al total filtration at a 50-cm FSD and a dose rate of 250 rad/min.

RESULTS

The effect of radiation-sterilized cells on the plating efficiency of viable cells was tested by plating 250 viable cells on 1-day-old feeder layers of different cell densities (Fig. 1). Feeder layers composed of 10^2 or 10^3 cells/dish had no effect on the plating efficiency. However, the plating efficiency increased as the density of the cells in the feeder layer was increased from 10^4 to 10^6 radiation-sterilized cells/dish. Feeder layers with higher cell densities were not tested because experiments using feeder layers of such high densities would have required prohibitive numbers of tumor cells.

In order to determine the effect of varying the time interval between plating the feeder layer and plating the experimental cells a cell suspension was prepared and plated on feeder layers of 10^6 radiation-sterilized cells/dish which had been prepared 4 days, 2 days, 1 day or 4 hr previously. The results of a typical experiment are shown in Fig. 2. In this and replicate experiments 1-day-old feeder layers gave the highest plating efficiencies. However, feeder layers 2–4 days old produced plating efficiencies only slightly lower than those obtained with 1-day-old feeder layers. There was

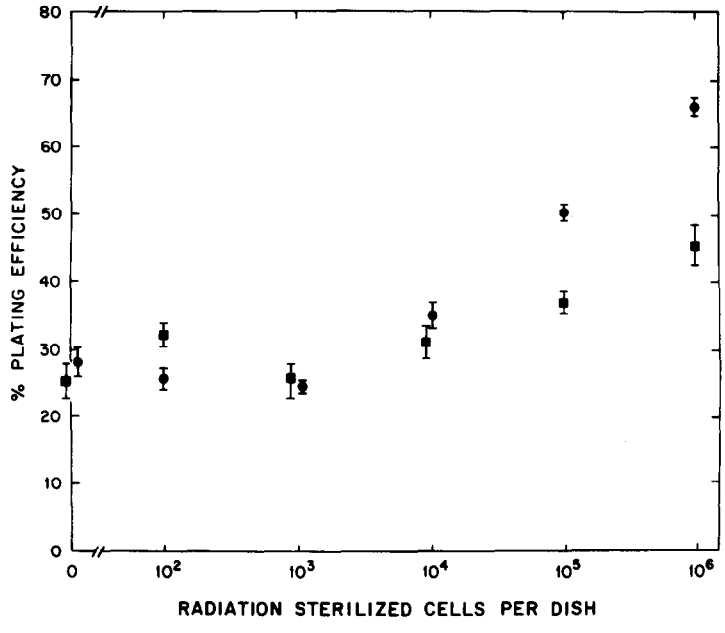

Fig. 1. Effect of feeder layer density on plating efficiency. Points are means with standard errors derived from colony counts on 5 replicate dishes. Different symbols represent separate experiments.

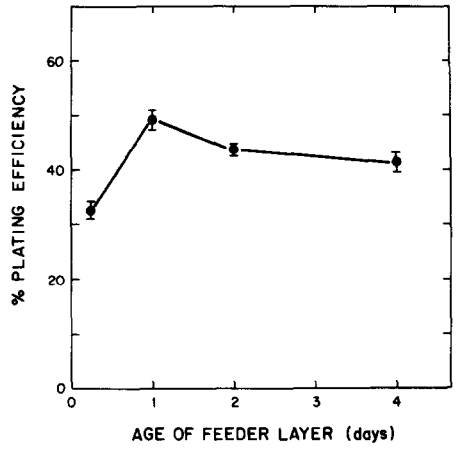

Fig. 2. A representative experiment showing the effect of feeder layer age on plating efficiency. Points are means with standard errors derived from colony counts on 5 replicate dishes. All groups were plated from a single tumor cell suspension.

no difference in the size or appearance of colonies grown on feeder layers of different ages. Feeder layers from 1 to 4 days old consistently produced plating efficiencies higher than feeder layers prepared on the day that the experimental cells were plated.

To determine whether the plating efficiency was dependent on the density of the experimental cells, tumor cells from a single suspension were plated at several different densities on a 1-day-old feeder layer composed of 10^4 radiation-sterilized cells/dish. As shown in Fig. 3, the plating efficiency did not change when the number of cells plated was varied over the range of 125–1250 cells/dish.

The effect of holding the cell suspension at various temperatures for up to 4 hr before plating was determined. A cell suspension was prepared as described above and an aliquot was diluted and plated immediately. The remaining suspension was then divided; one portion was held in a 37°C water bath and the other held at room temperature. After 1, 2 and 4 hr aliquots were again diluted and plated. There was no change in the plating efficiency when cells were held for up to 4 hr at either 37°C or room temperature (Fig. 4). The plating efficiency was also stable for up to 4 hr when the cells were held on ice (data not shown).

The plating efficiencies of different tumor cell suspensions tested over 2 yr in 40 different experiments varied from 30 to 80%, with a mean of 50%. To examine the possible sources of variation in the assay procedure an analysis of variance, assuming a random effects model, was carried out. The variance of a single observation (No. of colonies/cell) was assumed to be equal to the sum of the variances of three effects: (1) variation from experiment to experiment; (2) variation among tumors within an experiment; and (3) replicate (plate-to-plate) variation. The components of the variance for these effects were estimated as 0.010, 0.0020 and 0.0029 respectively. These estimates indicate that the experiment-to-experiment variation was approximately 5 times as large as the variation among tumors within a single experiment. The variation among tumors within an experiment was similar to the variation among plates from the same tumor. Because of the experiment-to-experiment variability in plating

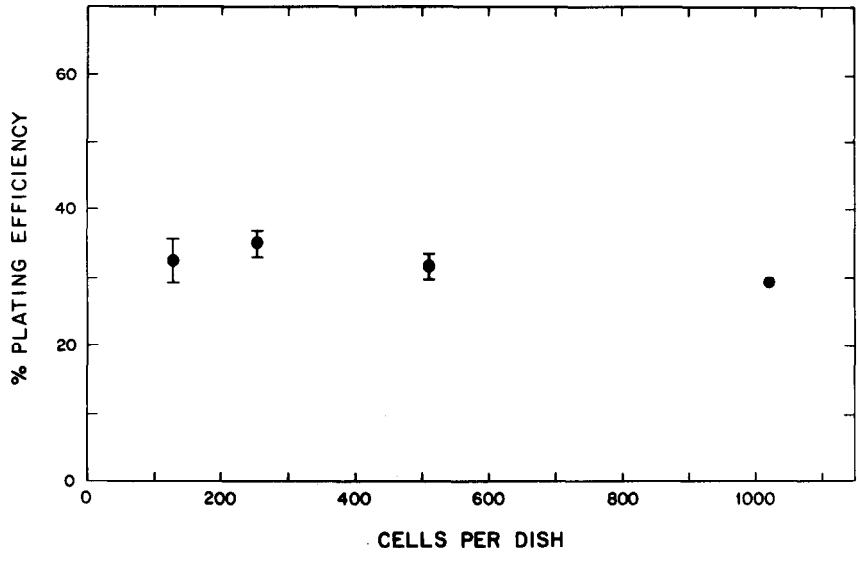

Fig. 3. A representative experiment showing the effect of viable cell density on plating efficiency. Varying numbers of freshly prepared cells were plated on a 1-day-old feeder layer composed of 10^4 cells/dish. *Points are means derived from colony counts on 5 replicate dishes. Standard errors are shown where larger than the symbols. All groups were plated from a single tumor cell suspension.*

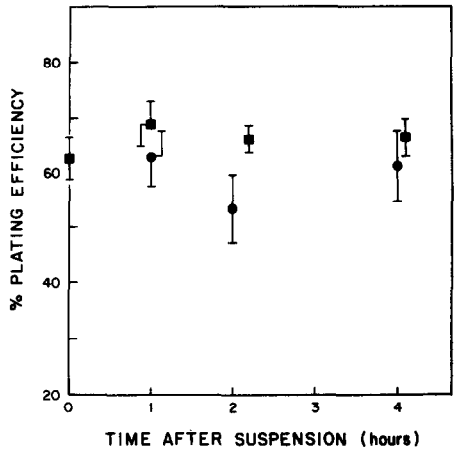

Fig. 4. A representative experiment showing the variation in plating efficiency with time between suspension and plating. Two hundred and fifty cells were plated on a 1-day-old feeder layer composed of 10^6 cells/dish, *either immediately after suspension or up to* 4 hr *later.* • *Cells held at room temperature between suspension and plating;* ■ *cells held at 37°C between suspension and plating. Points are means with standard errors derived from colony counts on 5 replicate dishes. All cells were from a single tumor cell suspension.*

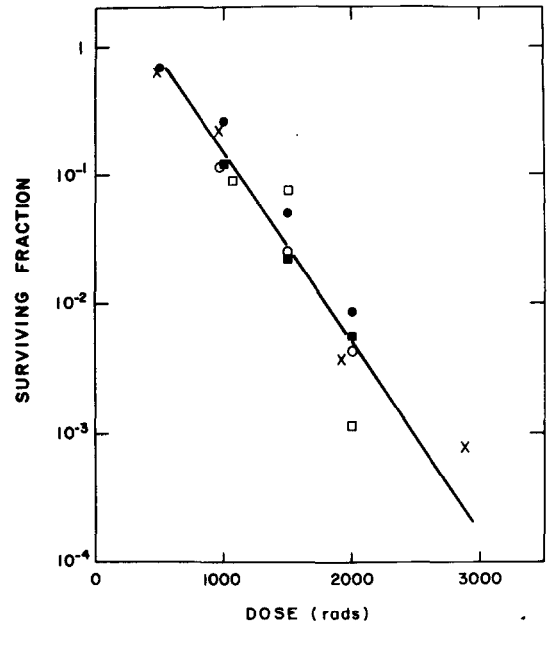

Fig. 5. Survival curve for cells suspended from tumors irradiated in air-breathing animals. Cell suspensions were prepared and assayed for viability immediately after irradiation. Each point is the surviving fraction for cells from a single tumor. Different symbols indicate separate experiments. X: surviving fractions determined by Reinhold using a tumor formation assay (replotted from 3). Line: least square regression line fitted to data at doses greater than 1000 rad.

efficiency surviving fractions for cells from treated tumors should always be calculated using the plating efficiencies of untreated control cells plated on the same day.

To determine whether the techniques described in this report could be used to assay the survival of cells treated with cytotoxic agents the survival curve was determined for cells from BA1112 tumors irradiated with different doses of X-rays in unanesthetized, air-breathing animals (Fig. 5). The terminal portion of the survival curve was linear and characterized by a D_0* of 290 rad (95% C.L. 230–410) and an intercept of 4 (95% C.L. 1–20). However, the intercept should not be considered to be a measure of the shoulder of the sur-

*The dose required to reduce the number of clonogenic cells by a factor of 1/e along the linear portion of the survival curve.

vival curve because the tumor contains both hypoxic and aerobic cells [10].

DISCUSSION

The BA1112 rhabdomyosarcoma has been used for more than 15 yr to study the response of tumors to therapy. Over this period the tumor has been remarkably stable and a large body of data has been collected regarding the control of the tumor by single-dose and multifraction radiotherapy, alone or in combination with drugs [4, 10–13]. This tumor is well suited for studies with *in situ* assays which use gross tumor response, such as tumor control, as the endpoint.

About 15 years ago Hermens and Barendsen adapted the BA1112 tumor for growth *in vitro* [14]. The tumor system which resulted, named R-1, is currently being studied in a number of laboratories. However, the characteristics of the R-1 tumor vary significantly from those of the BA1112. Moreover, the R-1 line has been unstable and there are now a number of R-1 sublines with different characteristics [15]. Therefore *in vitro* studies and cell survival measurements performed with R-1 cells and tumors cannot be used to interpret the results of *in situ* studies performed with BA1112 tumors. For this reason we decided to attempt to develop techniques which could be used to assay *in vitro* the survival of cells from BA1112 tumors. The BA1112 tumor has never been passaged *in vitro*, thus avoiding the possible selection process associated with *in vitro* cultivation [16].

An accurate cell survival assay must be insensitive to the presence of large numbers of dead or dying cells. In the BA1112 system the presence of a large number of radiation-sterilized cells was found to have a considerable effect on the plating efficiency of untreated cells (Fig. 1). Therefore all determinations of the viability of cells from treated tumors were carried out using a feeder layer composed of 10^6 heavily irradiated cells/dish prepared at least 1 day prior to plating the experimental cells. This technique improved the colony-forming ability of the experimental cells (Fig. 1) and eliminated potential artifacts due to the feeder-layer-like effects of cells killed by intensive treatments. At least a 10-fold excess of feeder cells over experimental cells was used in all survival determinations.

Cell survival measurements must not be affected by the number of clonogenic cells which are plated. The plating efficiency of untreated BA1112 cells was not altered by varying the number of experimental cells plated per dish from 125 to 1250 cells (Fig. 3). The plating efficiencies of cells from irradiated tumors were similarly insensitive to the plating density. In irradiation experiments several different cell densities were plated to ensure that at least one group with a countable number of colonies would be obtained. When countable numbers of colonies grew in 2 groups of dishes plated at different cell densities from the same irradiated tumor the measured surviving fractions were found to be similar in both groups.

The stability of the cell preparation is important for experiments which require lengthy manipulations of the cell suspension between the preparation of the suspension and the actual plating. The plating efficiency of untreated cells did not decrease during the first 4 hr after the preparation of the suspension when the cells were held in full medium at room temperature, at 37°C or on ice. During this period the cells did not attach to the tube but did form clumps which could be readily dispersed by vortexing the suspension.

The radiation survival curve for BA1112 tumor cells in air-breathing animals determined using the *in vitro* colony formation assay (Fig. 5) was similar to that obtained with this tumor by Reinhold [3]. The irradiation and cell suspension procedures were similar in both sets of experiments, but Reinhold measured cell survival *in vivo* using the TD_{50} assay [17]. The good agreement between these two sets of data makes it unlikely that the survival curve was seriously affected by the potential artifacts associated with colony formation *in vitro*. The similarity between these two cell survival curves, determined more than 15 yr apart, also implies a remarkable

Table 1. *Characteristics of* in vivo–in vitro *rat tumor systems*

Tumor	Origin	Immunogenicity	Metastasizes	Tumor control assay	Tissue culture adapted	*In vivo/in vitro* plating efficiency (%)	Hypoxic fraction	Reference
BA1112	Spontaneous	–	–	+	–	52	0.17	[10]
R-1	Spontaneous	–	–	+	+	18–35	0.35	[14, 15, 18]
9L	Induced	+	–	–	+	20–40	0.05	[19]
RIB5C	Induced	+	+	?	+	60–80	0.11–0.26	[20, 21]
R3326-AT	Induced	+	–	+	+	4	?	[22]
H-4-II-E	Induced	±	+	?	+	50–60	?	[23]

stability in the radiosensitivity of the tumor system. This stability is also evidenced by the consistency of the *in vivo* radiation response of the tumor over this same period [10].

The methods described in this communication make it possible to assay *in vitro* the survival of BA1112 tumor cells which have been treated *in vivo*. BA1112 has joined the growing list of tumors which can be treated *in vivo* then excised and assayed for cell survival *in vitro*. The characteristics of the existing *in vivo–in vitro* rat tumors are summarized on Table 1. Each of these tumors has a unique set of characteristics which suit it for a particular type of study. Among these tumors, BA1112 is unusually well suited to assays which measure tumor response *in situ*. The tumor has remained extremely stable over the years. Moreover, it is nonimmunogenic and does not metastasize; therefore the control or growth of tumors can be studied and used to quantitate and compare the effects of different therapeutic agents and regimens. Using the methods described in this report, it is now practicable to measure cell survival as well. The response of BA1112 tumors to radiation has now been measured using tumor control and cell survival assays. A comparison of the results obtained with these assays is described elsewhere [10].

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