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ANALYSIS OF TUMOUR RESPONSES BY EXCISION AND *IN VITRO* ASSAY OF CELLULAR CLONOGENIC CAPACITY

G. W. BARENDSEN

From the Radiobiological Institute of the Organization for Health Research TNO, 151 Lange Kleiweg, Rijswijk and the Laboratory for Radiobiology, University of Amsterdam, 121 Plesmanlaan, Amsterdam, The Netherlands

Summary.—A review is presented of the advantages and problems associated with the use of *in vitro* assays of cellular clonogenic capacity when used to analyse responses of tumours treated *in vivo*, either with radiation or chemotherapeutic agents. Three questions are considered:

(1) Does the cell suspension obtained by the dispersion technique from various types of solid tumours provide an adequately random sample of the cells initially present in the tumour?

(2) Are the properties of cells in suspensions obtained from solid tumours assayed in optimal conditions *in vitro*?

(3) Are the properties expressed and analysed by *in vitro* techniques equivalent to the properties that the same cells would have expressed if they had been left *in vivo*, either in untreated or in treated tumours?

It is concluded that the *in vitro* assay provides a valuable tool to analyse tumour responses but that the data obtained must be complemented by information on cell kinetics and other factors to obtain a complete description of tumour responses and to correlate them with tumour curability and growth delay.

THE ULTIMATE aim of studies on experimental solid tumours is a complete understanding of the mechanisms that determine malignant growth and reactions to various treatments, with a view to improve the treatment of cancer in man. For such a complete understanding it is necessary to analyse the clonogenic capacity and proliferation kinetics of various sub-populations of cells in the tumour and the changes after the administration of various agents (Barendsen, 1980). Solid tumours are not a homogeneous mass of malignant cells, however, but consist of a complex and heterogeneous cell population in a variety of physiological conditions. The processes and factors involved in tumour growth and reactions to different doses of various agents cannot be studied in detail by assessment of volume changes and curability alone. Therefore, investigations were started in the early 1960s to prepare cell suspensions from solid tumours with the aim of studying properties of these cells. The development of a quantitative cell dispersion technique for a transplantable solid tumour was published by Reinhold (1965). He employed the trypsinization technique with the BA1112 rat rhabdomyosarcoma for studies of the radiosensitivity of cells treated *in vivo* and analysed by the endpoint dilution assay, similar to the method published for a mouse leukaemia by Hewitt & Wilson (1959). The first studies in which solid tumours were excised and the derived cells were directly quantitatively assayed *in vitro* for cellular clonogenic capacity were reported for the R-1 rhabdomyosarcoma transplantable in WAG/Rij rats (Barendsen & Broerse, 1969). This tumour was obtained as a subline of the BA1112. Since then the *in vivo–in vitro* assay has been developed for at least 10 other transplantable tumours and these tumour cell culture systems are now being used for a variety of quantitative studies (Rockwell, 1977).

The *in vitro* assay for cell clonogenic

capacity was initially developed to obtain a more rapid and economical method than the endpoint dilution assay, where 40–70 syngeneic recipient animals, injected with different numbers of cells, must be kept and observed for tumour development for 3–6 months. Later developments have led to more rapid assays *in vivo*, which employ observations of tumour nodules developing in lungs of recipient animals, after intravenous injection of cells. These assays are discussed by Hill (1980). In addition to the assay for clonogenic capacity, *in vitro*-cultured tumour cells have been used to measure proliferation kinetics in tumour cell subpopulations using ^{3}H-TdR labelling techniques (Barendsen *et al.*, 1973; Hermens & Barendsen, 1978). Soft agar culture conditions have recently been applied to replace standard medium cultures in order to improve plating efficiencies for cells from some experimental tumours and for cells from human tumours (Courtenay *et al.*, 1976).

In the present contribution three problems associated with the *in vitro* assay of the clonogenic capacity of cells from experimental tumours will be discussed, namely:

(1) Does the cell dispersion technique applied to experimental solid tumours provide a suspension that is an unselected, representative sample of the original cell population in the tumour?
(2) Are the properties of cells in suspensions derived from tumours assayed in optimal conditions?
(3) Are the properties expressed and analysed by *in vitro* techniques equivalent to the properties that these cells would have expressed had they been left *in vivo*, either in untreated or in treated tumours?

The cell dispersion technique

The first step in the procedure required for the analysis of cellular responses of tumours after excision, is the application of a dispersion technique. The disaggregation procedure with trypsin developed by Reinhold (1965) has been used for many tumour systems, although recently the application of an enzyme "cocktail" containing pronase, DNAse and collagenase has been reported to provide a larger cell yield/g of tumour for the EMT6 mouse tumour (Brown *et al.*, 1980).

An important problem associated with the preparation of cell suspensions from tumours is that not all tumour cells are quantitatively recovered. This implies that a selection of the original population of cells might be obtained in the suspension, and that the analysis of properties of these cells does not necessarily provide information on the characteristics and responses of all cells originally present in the tumour.

In order to reduce the magnitude of this problem it is important to attain a high cell yield. For the R-1 rhabdomyosarcoma we have regularly obtained yields of 10–50×10^6 cells/g of tumour. Since this tumour contains about 5×10^8 cells/g, the yield of the dispersion technique is in the range of 2 to 10% (Barendsen & Broerse, 1969). For the other tumour system widely used in many laboratories for *in vitro* analysis, namely the EMT6 mouse tumour, the cell yield is somewhat lower (approximately 1%). This has been increased to about 10% by using the enzyme "cocktail" instead of trypsin. For other tumours, values of the cell yield ranging between 1 and 35% have been reported, but in no case have values approaching 100% been attained (Twentyman *et al.*, 1980; Dawson *et al.*, 1973; Stephens & Peacock, 1978). Thus, the possibility cannot be excluded that the cells obtained in suspensions from solid tumours are not an adequate random sample of the cells initially present in the tumours. Therefore, studies must be performed to analyse whether various subpopulations of cells are proportionally represented.

Several subpopulations of cells in a tumour can be distinguished according to various characteristics. Firstly, tumours contain both malignant cells and cells of

normal origin which form blood vessels and other structural elements. For some tumours the malignant cells have a different DNA content from normal cells, and this can be analysed by flow cytofluorimetry techniques. However, an increased DNA content is by no means a general, characteristic difference between tumour cells and normal cells (Kal, 1973*a*).

A further problem is that cells in tumours may be present in a resting phase (G_0-phase). This cell state may be caused by conditions in the micro-environment, *e.g.* inadequate supply of oxygen and nutrients, but other tumour characteristics, *e.g.* differentiation properties, might also influence the fraction of noncycling cells. Cells in different proliferative states may be differentially susceptible to damage by the trypsinization procedure and this may result in selection. In addition, it is known that tumours may contain fractions of hypoxic cells. If lack of oxygen or nutrients causes cells to be in a nonproliferating phase, a correlation between hypoxic fractions and the proportion of cells in G_0 would be expected. Finally, among the population of cells in progress through the proliferative cycle, cells in G_1, S, G_2 and M can be distinguished (Kal, 1973*b*).

It is evident that due to the elaborate techniques involved, it is not possible to analyse, in all experiments with cells from excised tumours, whether cells from any of the subpopulations are preferentially lost due to the disaggregation procedure. Nevertheless, for each type of tumour to be studied with respect to its response to radiation or drugs, at least some experiments on this problem should be carried out, using *e.g.* labelling of cells with ^{3}H-TdR to distinguish G_0, G_1, S and G_2 cells or employing flow cytofluorimetry techniques.

With the R-1 tumour, repeated injections of ^{3}H-TdR at 4-h intervals have been used to label all proliferating cells and to distinguish these from nonproliferating cells. After the last injection tumours were excised, cell suspensions prepared and cells cultured for different time intervals up to 120 h. The labelling index of cells in 12-h cultures was 0·48, which was not significantly different from the value of 0·51 for cells in the tumour. This indicates that the cell dispersion technique employed to prepare cell suspensions from these R-1 tumours did not preferentially select proliferating or nonproliferating cells (Barendsen *et al.*, 1973). A similar conclusion could be drawn concerning G_1, S or G_2 phase cells. Comparable data have been obtained for the EMT6 tumour system (Kallman, 1979).

In a later series of experiments with larger R-1 tumours, however, the labelling index in the tumour was somewhat lower than that of 12-h cultures of cells from suspensions prepared from the tumour. This might be considered as evidence that a fraction (30–40%) of the nonproliferating cells show a reduced capacity for recovery from damage due to the trypsinization procedure and do not attach to the culture dishes (Hermens & Barendsen, 1978).

After treatments of tumours with ionizing radiations, chemotherapeutic drugs or other agents, the cell yield has been reported to decrease for various types of tumours (Barendsen & Broerse, 1969; Kal and Barendsen, 1980; Stephens & Peacock, 1978; Rockwell, 1977; Steel *et al.*, 1977). This decrease in yield may vary from a factor of about 2 for relatively low doses of radiation or drugs to factors as high as 20 for fractionated treatments (Barendsen & Broerse, 1970; Stephens & Peacock, 1978). For the R-1 tumour a decrease in yield by a factor of about 2 at 1–2 days after irradiation with 1000 rad of X-rays could be accounted for by assuming that the mean volume of the cells increases. This increase would be expected for cells that experience a mitotic delay due to their treatment. However, the yield of cells from R-1 tumours treated with fractionated doses over several weeks decreased much more than a factor of 2 and this was ascribed to an enhanced susceptibility to lysis by prolonged expo-

sure of cells to trypsin during the dispersion procedure. Stephens & Peacock (1978) described differences in cell yield after treatment of B16 melanomas by various cytotoxic agents. After treatments with radiation and several alkylating agents yields were reduced by about 40%, associated with an increase in cell volume. With Vinca alkaloids, however, a larger reduction in yield was associated with extensive cell lysis, observed as an increased fraction of necrotic cells. In contrast, 5 FU increased the yield of cells from these tumours by 30%.

It must be concluded that cell yield is an important parameter to measure if the effect of various treatments of tumours *in vivo* is to be assessed *in vitro* and that variations by at least a factor of 2 and sometimes much larger are frequently observed. Since measurements of cell yield show variations by a factor of 2 even for untreated tumours, it is evident that differences by a factor of 2 in cell survival measured after a given treatment are difficult to establish with accuracy. Furthermore, much more work on this phenomenon is required to determine the factors and mechanisms involved.

In vitro *growth conditions*

After disaggregation of a tumour and preparation of a cell suspension, cells have to be cultured in conditions which are optimal for the expression of the properties being investigated. Since the capacity for unlimited proliferation is the characteristic of greatest significance in cancer therapy, assessment of clonogenic capacity is generally measured, rather than proliferation rate, uptake of proteins or other parameters. Nevertheless it might well be worthwhile to pay more attention to the metabolism and proliferation kinetics of tumour cells shortly after they have been removed from tumours, because their characteristics would be expected to reflect the state and condition of the cells in the tumour and this might provide additional interesting information on tumour growth and responses to treatments (Barendsen *et al.*, 1973; Hermens & Barendsen, 1978).

With respect to the problem of optimum *in vitro* conditions for clone formation, the first criterion to be considered is the plating efficiency of cells from untreated tumours. For cells propagated in culture, plating efficiencies between 60 and 100% are frequently observed, but for cells directly derived from tumours, the corresponding values are generally lower. For the R-1 tumour and the EMT6 tumour values are usually between 30–40% but for other tumours values between 1 and 80% have been reported (Rockwell, 1977). The lower plating efficiencies for cells taken directly from tumours do not necessarily reflect inadequate culture conditions, however. It is quite possible that in the tumour a fraction of cells already exists in unfavourable conditions, *e.g.* due to lack of oxygen or nutrient supply. These cells, if not killed by the trypsinization technique, may be present in the suspension but might not be expected to produce clones *in vitro* with a high efficiency.

Information about whether culture conditions are adequate may be derived from a comparison of the sensitivity to various agents obtained by assays *in vitro* and *in vivo*. Comparisons of survival curves of cells from the Lewis Lung Carcinoma after treatment with single doses of cyclophosphamide, assayed by the *in vitro* cloning technique, by the endpoint dilution technique and the lung colony technique showed good agreement (Steel & Adams, 1975). For other tumours, notably R-1 and EMT6, agreement between colony formation assays and endpoint dilution assays has also been observed (Barendsen & Broerse, 1969; Rockwell, 1977).

It should be noted, however, that it is important to study quantitatively the influence of the culture conditions for each tumour, *e.g.* using different numbers of feeder cells, conditioned medium and different serum concentrations, in order to assess the dependence of cell survival on these factors. It is also important to be aware of the possible influence of artefacts,

such as the carry-over of drugs from tumours to culture conditions, that might influence the clonogenic capacity of the cells to be assayed (Twentyman, 1977). In general, it can be concluded that if plating efficiencies in excess of 30% are obtained for cells from untreated tumours, culture conditions would not be expected to be of critical importance for the analyses of tumour responses to various treatments by *in vitro* assays. However, even with lower plating efficiencies it is possible that the culture conditions are adequate for use of the system in quantitative tumour response studies.

A development of particular interest is the use of soft agar techniques possibly allowing direct cloning of unselected primary animal tumours and hopefully of human tumours (Courtenay *et al.*, 1976).

Significance of in vitro *assay for analyses of tumour responses*

The ultimate test of the validity of data obtained by *in vitro* assay of the reproductive integrity of cells from tumours is the correlation with responses of tumours measured by growth delay and with fractions of tumours cured by a given treatment. This correlation is not always a direct one and we cannot necessarily expect *in vitro* assays for clonogenic capacity to provide all the answers about tumour responses *in vivo*. However, the information obtained by *in vitro* studies of tumour cells provides insights that cannot be obtained by other methods. Therefore, it is of great importance to extend such studies to other tumours and other treatments, without, however, ignoring the limitations inherent in their application. Some of these limitations will be discussed briefly.

It is evident that cells remaining in tumours after treatment will be maintained and will proliferate in an environment that is quite different from *in vitro* culture conditions. The factors include intercellular contact, the supply of oxygen and nutrients, proliferation stimuli from the host, immunological reactions of the host, the continued presence of drugs, tumour shrinkage, the presence of lysed cells and cell debris, damage to normal structures, *e.g.* blood vessels and other elements collectively denoted tumour bed effects, and possibly others, as summarized in the Table.

TABLE.—*Factors influencing the survival and proliferation of cells in tumours after treatments*

1. Repair of potentially lethal damage
2. Oxygen supply and utilization
3. Intercellular contacts with surviving and lysing cells
4. Nutrient supply
5. Changes in blood vessels and structural elements (tumour shrinkage, tumour bed effect)
6. Local proliferation stimuli of the host
7. Immunological reactions of the host
8. Continued presence of drugs

With respect to repair of potentially lethal damage it is well known that damage by ionizing radiation and by some drugs applied in short exposures, is not immediately fixed and can be modified by changes in conditions of cells after exposure. The period during which fixation of damage occurs has been shown in several systems to last about 4–6 h. Therefore, it is important to excise tumours not less than 4–6 h after irradiation unless evidence has been obtained that this factor is of little significance, as was observed for the R-1 tumour (Barendsen & Broerse, 1969).

With respect to the influence of hypoxia, it is evident that excision of the tumour and preparation of the cell suspension renders all cells oxygenated. The time interval between tumour treatment and tumour excision should play a part in the interpretation of responses assayed *in vitro* (McNally, 1972; Courtenay *et al.*, 1978).

In the case of treatments with chemotherapeutic agents it is evidently also of great importance at what interval after administration the assay is performed, because the action of these agents may continue for a long period, *i.e.* several days. This problem has been discussed by

several authors (Twentyman, 1978, 1979; Hahn *et al.*, 1973; Kal *et al.*, 1979; Kal & Barendsen, 1980.)

Finally, it is important to discuss the immunogenic differences between a tumour and its host. It is well known that these differences may complicate the analysis of tumour responses to treatments and studies performed with immunogenic tumour systems should be interpreted with the utmost caution. This problem has been discussed in detail by Rasey *et al.* (1977) and Rasey & Nelson (1980).

Notwithstanding all these problems concerning insufficient knowledge about the influence of various factors, it is of interest to note that quite good agreement has been obtained in many instances between data obtained by *in vitro* analysis of cell clonogenic capacity and tumour curability. An example of this is shown in the Figure for the R-1 tumour treated with single doses of X-rays and with multiple fractions of 300 rad/day. A similar conclusion has been obtained for the Lewis Lung Carcinoma (Steel & Adams, 1975) and for tumour SSK-31 (Trott, personal communication).

Comparison of cell survival assayed *in vitro* and growth delay data frequently shows less satisfactory agreement. This must be ascribed to the fact that growth delay is influenced by many factors which alter the cell proliferation rate, notably removal of lysed cells and acceleration of repopulation. Thus, it is not surprising that RBE and OER values, as well as parameters associated with repair, assessed from growth delay measurements may lead to erroneous conclusions (McNally, 1973). Differences between cell killing effectiveness and growth delay have also been observed for various chemotherapeutic agents (Begg *et al.*, 1980).

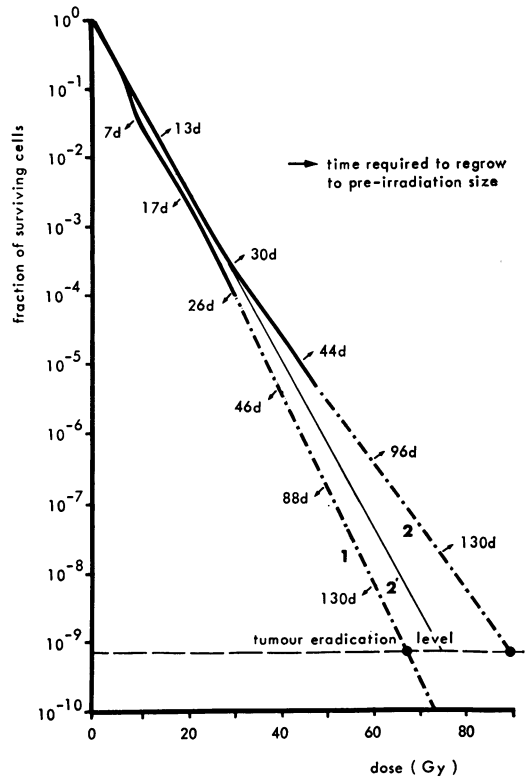

FIGURE.—Relationships between fractions of clonogenic cells present in R-1 rhabdomyosarcomas and doses of 300 kV X-rays administered as single doses (curve 1) or doses of 3 Gy/fraction given 5 days/week at 24-h intervals (curve 2). Curve 2′ is the extrapolation of the region of curve 2 up to 30 Gy administered in 2 weeks. After 2 weeks of treatment with 3 Gy/day, an accelerated proliferation causes a change in slope of curve 2 at doses in excess of 30 Gy. For various doses corresponding to the origins of the arrows, the tumour growth delays are given in days. The level of survival equal to $0{\cdot}7 \times 10^{-9}$ is indicated at which, for a tumour that contains $1{\cdot}5 \times 10^{8}$ clonogenic cells at the start of the treatment, the probability of one clonogenic cell remaining after treatment is reduced to 0·1. This corresponds to expected TCD_{90} values of 67 Gy and 90 Gy for curves 1 and 2 respectively. The corresponding observed values were 65 Gy and 90 Gy respectively (Barendsen & Broerse, 1970).

Concluding remarks

In conclusion it can be said that *in vitro* assays of the clonogenic capacity of cells from tumours treated *in vivo* can provide valuable information about the effectiveness of various agents on tumours with respect to cell killing *in vivo* and the prediction of curability, but that the data should be supplemented by information on cell proliferation kinetics and other factors mentioned in the Table, in order

to provide a good correlation with tumour growth delay.

Finally, it is of interest to mention briefly a new development which has recently been explored to apply *in vitro* analysis techniques to the study of responses of tumours. The *in vitro* assay for cells from tumours was introduced initially to provide a more rapid assay of clonogenic capacity for cells from dispersed tumours than the endpoint dilution assay. Reproducible assays have been obtained for transplantable tumours, but for primary tumours in animals and in man the method is not yet applicable because of low plating efficiencies for controls. Recent developments with soft agar techniques should be developed further to overcome these problems, but even with improved culture conditions, analysis of the clonogenic capacity of cells from primary tumours will take several weeks before the effectiveness of treatments can be assessed.

In order to develop a method that might yield insight into the effectiveness of treatments in a shorter time interval, Drs Aten and Kipp have recently attempted to correlate damage to the cells' reproductive capacity with damage to chromosomes using flow cytometry techniques applied to chromosome suspensions from irradiated cells (Aten *et al.*, 1980). Cells in culture or tumours in animals were treated with vinblastine to arrest cells in mitosis and 8 h later chromosome suspensions were prepared. DNA content histograms were measured by means of flow microfluorimetry using a Biophysics FC 4800A-FC 200 system. In the histograms chromosomes are represented by separate peaks. The shapes of the peaks are altered as a result of irradiation, because of the appearance of damaged chromosomes. These changes have been analysed quantitatively by the application of fourier analysis. If this method for cells in culture can be applied quantitatively to tumours after irradiation or drug administration, the effectiveness of treatments could be assessed within 24 h. This would allow an even more rapid analysis than can be obtained by *in vitro* clone formation or lung colony assays. This technique, if it could be developed for application to human tumours, might eventually yield a rapid method for assessment of drug effectiveness in clinical applications.

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