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Induction of lethal mutations in experimental tumours after single and fractionated irradiations *in vivo*

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Abstract.
Purpose: To investigate the prolonged reduction in cellular viability (lethal mutations) of surviving cells following irradiation of tumours *in vivo* and to test the effects of fractionation on the expression of lethal mutations.
Materials and methods: A mouse mammary carcinoma (CaNT) was treated with single dose or fractionated X-ray treatments *in vivo* and survival quantified with an *in vitro* excision assay soon after irradiation and at various times up to 35 days after *in vitro* propagation of the surviving cells.
Results: A dose-dependent reduction in the plating efficiency was observed in cells isolated from irradiated tumours up to 35 days and many cell generations after irradiation. Considerable heterogeneity in plating efficiency was observed in clonal cell lines isolated from individual colonies grown from irradiated tumours. Delayed expression of lethal damage was observed after fractionated irradiation, although recovery of cellular fitness was greater than after irradiation with single doses (reported previously) suggesting that this form of damage is affected by inter-fraction repair. At equi-toxic doses, delayed expression of lethal damage was similar after three compared with two fractions of radiation per day (reported previously).
Conclusions: These effects indicate that conventional excision assays of tumour cell viability under-estimate the total lethal damage caused by irradiation and have implications for modelling of the response of tumours to radiotherapy. The effect of fractionation on expression of this type of damage implies the involvement of repair processes. Therefore the repair proficiency may affect the balance between the immediate and delayed reduction of viability in irradiated cells.

1. Introduction

A dose-dependent persistent reduction in colony-forming ability has been demonstrated among the progeny of irradiated cells when compared with non-irradiated cells. This reduction in cellular 'fitness' has been referred to as delayed expression of lethal mutations (LM), and has been observed in several *in vitro* studies with mammalian cell lines (Seymour *et al.* 1986, Mothersill and Seymour 1987, Gorgojo and Little 1989, Little *et al.* 1990) and recently with murine CaNT-tumour cells *in vivo* (Chatterjee *et al.* 1995). Accumulation of LM in cells from tumours exposed to many small fractions indicates that this damage occurs at relatively low doses of radiation and can build up over a period of time. Evidence is now accumulating that cells initially surviving irradiation and capable of proliferation may produce some descendants in which *de novo* chromosome aberrations (Kadhim *et al.* 1992) and gene mutations (Little *et al.* 1990) arise. Recently radiation induced instability in human bone-marrow cells and delayed apoptosis in the clonal progeny of sub-lethally irradiated stem cells was also reported (Kadhim *et al.* 1995) and similar effects have been observed *in vivo* (Watson *et al.* 1996, 1997). By considering expression of these LM among the survivors of the irradiated cells, the conventional survival curve has been corrected for the over-estimation of survival that occurs in the conventional Puck and Marcus assay (Puck and Marcus 1956, Seymour *et al.* 1986, Alper *et al.* 1988). Such late survival curves differ from the conventional survival curves mainly by exhibiting a reduced shoulder, which is an indication of induction of LM within the dose range encompassing the shoulder. However, there is no evidence for a reduced shoulder in the corrected single and fractionated dose survival curves from the CaNT tumour model (Chatterjee *et al.* 1995).

Induction of lethal mutations is dose-dependent and has been reported to be reduced by fractionation of the radiation dose *in vitro* with an inter-fractionation interval of more than 2 h (Seymour and Mothersill 1989). However, accumulation of lethal mutations in cells from tumours treated with up to 10 small fractions (Chatterjee *et al.* 1995) indicated that even with a 6 h inter-fraction interval which is sufficient for complete repair of damage to occur, residual damage from relatively small radiation doses (3·4 Gy) could accumulate over a period of time.

Excision and *in vitro* clonogenic growth of experimental tumours is a useful method of assessing the effect of a treatment on the viability of the cells within the tumours, but the induction of LM may affect the interpretation of the results. The potential contribution of LM to the *in vivo* response to radiation

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is worth investigating because of their relevance to radiotherapy and to excision assays for the intrinsic radiosensitivity of cells from tumours and normal tissues. Delayed expression of lethal damage means that conventional assays based on plating cells at a single time after irradiation underestimate the amount of damage attributable to a treatment, and this effect may not be constant with different cell lines or tumour types or with different treatment protocols. Mathematical analysis suggests that LM could potentially make a significant contribution to normal tissue damage by radiation (O'Reilly *et al.* 1994, Hendry and West 1995) although Hendry and West concluded that there was little experimental evidence from which to judge their actual contribution to normal tissue reactions. In order to establish the relationships between total dose and dose per fraction for induction and expression of lethal mutations in tumours, this study has evaluated lethal mutations in cells surviving after irradiation of tumours with three fractions per day and compared these data with those obtained previously after one or two fractions per day (Chatterjee *et al.* 1995). Analysis of cell lines derived from single surviving colonies has been used to examine the inter and intra-tumoural variability in expression of delayed expression of lethal damage.

2. Materials and methods

The poorly differentiated CaNT mouse tumour with a volume doubling time of about 3·5 days, is a mammary carcinoma of spontaneous origin (Hewitt *et al.* 1973). It was implanted as a cell suspension, subcutaneously into the rear dorsum of anaesthetized 3-month old male CBA mice. Tumours were selected for use at mean geometric diameters of 6 ± 1 mm calculated from three orthogonal measurements. The studies were carried out according to the regulations stipulated by the UK Animals (Scientific Procedures) Act 1986.

2.1. *Irradiations*

The protocol for irradiation of tumours and the plating and propagation of tumour cells from single surviving colonies, sorted aneuploid G_1 cells or from bulk cell populations is shown in figure 1. Unanaesthetized mice bearing tumours were irradiated in special lead jigs with 240 kVp X-rays filtered with 0·25 mm Cu and 1·0 mm AL (HVL 1·3 mm Cu) at a dose rate of 3·9 Gy min^{-1}. Groups of six mice supplied with free-flowing air were treated either with single doses (20 Gy) or with three daily fractions of radiation (2·34 Gy) separated by inter-fraction

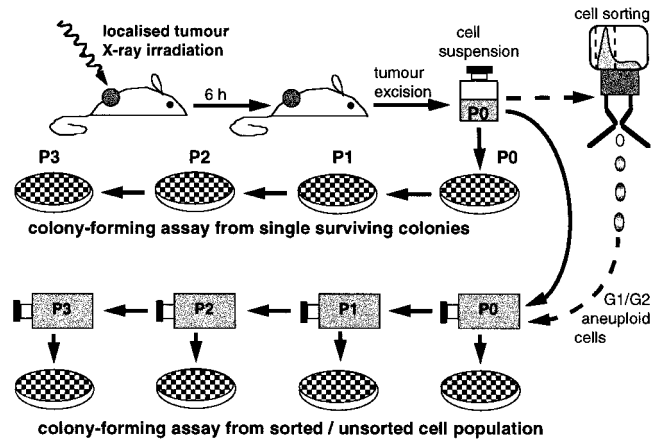

Figure 1. Experimental protocol for observing delayed expression of lethal damage in cells disaggregated from CaNT tumours following treatment *in vivo* with single or fractionated doses of radiation. Cells were propagated either from sorted/unsorted cell populations or from individual surviving colonies and their viability measured by plating at various times after the irradiation.

intervals of 6 h to allow complete repair of reparable damage to occur, for a total of 3, 6, 9, 11, 13 or 15 fractions.

2.2. *Excision assay*

In order to allow repair of potentially lethal and sub-lethal damage induced by irradiation, tumours were excised aseptically 6 h after treatment. A single cell suspension was prepared from each tumour as described previously (Chatterjee *et al.* 1995). Briefly, the minced material was stirred for 30 min at 37°C with 1 mg ml^{-1} pronase, 0·5 mg ml^{-1} collagenase and 0·5 mg ml^{-1} DNAase, dissolved in growth medium without serum. The enzymes were separated by centrifugation, a single cell suspension made, counted by Coulter counter and cells plated at two cell densities onto four 9 cm petri dishes each that had been prepared with a feeder layer of 2×10^5 lethally irradiated (30 Gy, aerobic conditions) V79 379A Chinese hamster cells. For unirradiated controls, six dishes were plated at 1 cell density. Dishes were incubated under an atmosphere of 5% CO_2+5% O_2 at 37°C for 12–14 days before fixing and staining the colonies with 0·2% crystal violet in 70% ethanol.

2.3. *Delayed plating of tumour cells*

In addition to immediate plating of tumour cells for survival estimation, cells from each tumour were propagated in flasks with feeder layers for subsequent plating efficiency assays. At the time of each assay, the cells which were being propagated were tryp-

sinized (0·05% trypsin, 0·02% EDTA) and plated at 2 cell densities on 9 cm diameter dishes and into 25 cm^2 flasks, at the higher cell density, to propagate them for subsequent assays. Each passage was of sufficient time (12–14 days) for colony formation to have occurred on the dishes plated in parallel at the start of each passage, and the flasks of cells were always sub-confluent.

2.4. *Tumour cells propagated from aneuploid* G_1 *sorted cells*

Freshly disaggregated cells from six individual tumours were stained with Hoechst 33342 (5 $\mu g\,ml^{-1}$) for 20 min at 37°C. Aneuploid cells were separated from the diploid stromal component of the tumours using a Becton Dickinson Vantage fluorescent activated cell sorter with an argon laser operated at 363 nm. The bivariate distribution of forward versus side scatter was used to pre-select single cells and reject debris and cell clumps, and a marker was set on a histogram of the DNA content (Hoechst fluorescence) of this population to define the aneuploid cells for sorting. The sorted cells were washed with growth medium and counted by haemocytometer and appropriate numbers from each tumour were plated onto 9 cm dishes without a feeder layer and in 25 cm^2 flasks for propagation of cells for subsequent estimation of plating efficiency as above.

2.5. *Tumour cells propagated from individual colonies*

In addition to bulk re-plating of cells, several cell lines were isolated from individual colonies surviving after immediate plating of tumour cells. Eight individual colonies of normal size (2–3 mm diameter) and appearance, which represented the majority of colonies on these dishes, were carefully scraped from separate petri dishes into small sterile tubes and the cells trypsinized (0·05% trypsin, 0·02% EDTA), washed and counted by haemocytometer. Appropriate numbers of cells were plated at a single cell density onto three 5 cm dishes. After 10–12 days growth, single colonies of normal appearance were selected by scraping from one of the dishes in each group and used to re-seed a further three dishes. This procedure was repeated a third time after another 10–12 days. Each time all the dishes were stained and used to assess plating efficiencies for the replated cells.

2.6. *Data analysis*

Logarithmically transformed survival data were fitted by unweighted least squares, using the RS1 package (BBN Software Products Corporation) implemented on a Microvax computer, to the linear-quadratic equation:

$$\ln S = -\alpha \times D - \beta \times D^2 \qquad (1)$$

The data derived from the immediate plating of tumour cells were corrected for the contribution of the reduced clonogenicity of their progeny by multiplying surviving fractions from day 0 (SF_0) by the surviving fractions (SF_p) obtained from subsequent passages:

$$SF_{0-p3} = SF_0 \times SF_{p1} \times SF_{p2} \times SF_{p3} \qquad (2)$$

Corrected survival data were plotted against day of plating for each dose point. To compare the effects of different numbers of fractions per day, a ratio (R) of initial: corrected survival was plotted against the survival data from conventional immediate plating:

$$R = \frac{SF_0}{SF_{0-p3}} = \frac{1}{SF_{p1} \times SF_{p2} \times SF_{p3}} \qquad (3)$$

The logarithmically transformed survival ratios and survival data were fitted to the equation:

$$R = \frac{1}{k \times SF_0} \qquad (4)$$

using the method of least squares, where k, p are constants. Higher values of R imply a larger difference between immediate and corrected survival and therefore a greater burden of lethal mutations.

3. Results

Approximately 10^7 clonogenic cells ($2-3 \times 10^7\,g^{-1}$) were obtained from untreated CaNT tumours and recovery of total cells was invariate with dose or survival level (data not shown). Clonal cell lines were derived from individual colonies of normal size and appearance grown from untreated tumours (table 1, figure 2) and from tumours treated with 20 Gy X-rays (table 1, figure 3) and their plating efficiencies assessed at several times. In control unirradiated cell lines (figures 2 and 4A) no consistent time-dependent trends were observed in the individual plating efficiencies, but all those derived from irradiated tumours showed a general time-dependent increase in plating efficiency (figures 3 and 4A). Some of the increase in plating efficiency between the immediate plating on day 0 and the first replating on day 11 could in principle reflect loss of normal stromal cells and lethally irradiated cells. However, the majority of the cultures harvested from individual colonies on day 11 will be of single cell origin and considerable differences were observed between the individual plating efficiencies and rates

Table 1. Number of cells plated and the plating efficicency observed at each passage for clonal cell lines derived from irradiated and unirradiated individual tumours.

	Day 0			Passage 1				Passage 2			Passage 3		
Sample	Cell no.	P.E. ± s.e.m.		Cells[a] /colony	Cell[b] no.	P.E. ± s.e.m.		Cell no.	P.E. ± s.e.m.		Cell no.	P.E. ± s.e.m.	
Unirradiated controls													
A	1650	0.293		1923	537	0.306		508	0.197		396	0.204	
B	1134	0.427		9200	467	0.135		389	0.358		193	0.213	
C	1272	0.230		1827	243	0.137		758	0.406		286	0.180	
D	1272	0.156		1625	267	0.189		909	0.362		347	0.224	
E	1300	0.244		3102	371	0.138		624	0.286		–	–	
F	1200	0.311		2184	406	0.159		796	0.296		194	0.174	
mean	1305	0.277 ± 0.037		3311	382	0.177 ± 0.027		664	0.317 ± 0.03		283	0.200 ± 0.010	
Irradiated with 20 Gy single dose													
G	1.11×10^5	0.00080		4219	578	0.0071		1154	0.193		529	0.185	
H	9.60×10^4	0.00043		7145	992	0.0013		741	0.141		543	0.390	
I	1.04×10^5	0.00037		4141	750	0.0011		816	0.293		598	0.216	
J	9.60×10^4	0.00031		5469	727	0.0032		821	0.195		544	0.214	
K	1.32×10^5	0.00041		11830	2180	0.0400		754	0.107		546	0.335	
L	1.06×10^5	0.00020		6965	1858	0.0550		892	0.195		582	0.292	
mean	1.07×10^5	0.00042 ± 0.00008	$p=0.0001$	6628	1181	0.0179 ± 0.01	$p=0.0001$	863	0.187 ± 0.026	$p=0.0042$	557	0.272 ± 0.0033	$p=ns$

[a]number of cells per colony isolated for the first time from day 0 plating.
[b]mean number of cells plated from 8 different colonies selected from previous passage.
p significance of difference between mean plating efficiencies for control and 20 Gy cell lines.
ns not significant.

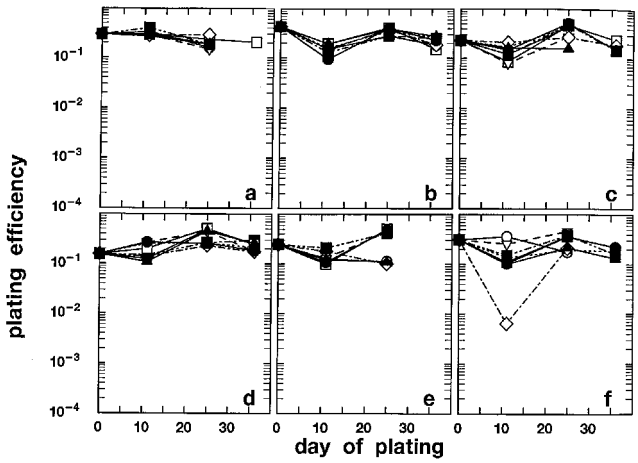

Figure 2. Time-dependent changes in plating efficiency of cell lines isolated from individual single colonies derived from unirradiated tumours excised, disaggregated and grown *in vitro* (plating efficiency day 0). Each panel represents data for eight different cell lines isolated from individual colonies (plating efficiency day 11) from a separate CaNT tumour and replated from single colonies at 25 and 36 days after excision of the tumour.

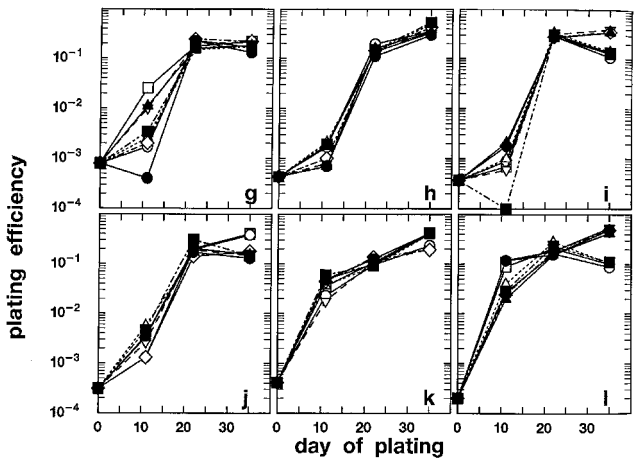

Figure 3. Time-dependent changes in plating efficiency of cell lines isolated from individual single colonies derived from tumours treated with 20 Gy X-rays, excised, disaggregated and grown *in vitro* (plating efficiency day 0). Each panel represents data for eight different cell lines isolated from individual colonies (plating efficiency day 11) from a separate CaNT tumour and replated at 22 and 35 days after excision of the tumour.

of recovery in plating efficiency of such clonal cell lines both within groups derived from single tumours (e.g. figure 3g) and between groups derived from different tumours (e.g. figures 3h and 3i versus figures 3k and 3l).

Similar trends were observed when aneuploid G_1 tumour cells were individually sorted from six tumours and the populations propagated separately in flasks plated 0–35 days after irradiation (figure 4B).

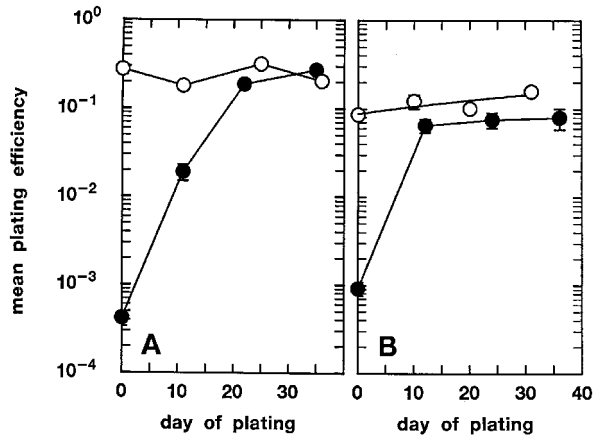

Figure 4. Time-dependent changes in mean plating efficiency of: (A) clonal cell lines isolated from single colonies derived from (○) untreated (figure 2) and (●) irradiated (20 Gy) (figure 3) CaNT tumours. (B) the G_1 aneuploid component from six (○) untreated and six (●) irradiated (20 Gy) CaNT tumours separated by cell sorting. Error bars indicate standard errors of the arithmetic mean and where not shown, are smaller that the size of the points plotted.

As these cells were plated without feeder layers, the overall control plating efficiency was lower than in the rest of this work. In this experiment, recovery in plating efficiency can not be ascribed to elimination of stromal cells or feeder cells. However, a persistent long-term reduction in the plating efficiency of irradiated compared with unirradiated cells was observed which was significant after passage 1 ($p=0.02$) and passage 3 ($p=0.007$) but failed to reach significance after passage 2 ($p=0.09$).

A persistent reduction in plating efficiency was also observed after administration of several fractions of radiation ($3 \times 2{\cdot}34$ Gy/day), with the greatest effect accumulating after 13–15 fractions (figure 5). The effect of this fractionation protocol on generation of a persistent reduction in plating efficiency was compared with data previously obtained from CaNT tumours treated with single doses or with twice-daily fractionated radiation (Chatterjee *et al.* 1995; table 2, figure 6). The corrected survival data for the three treatment protocols demonstrates a persistent reduction in cellular viability after both single-dose (figure 6A) and fractionated (figure 6B and C) irradiations, but it is not clear from figure 6 whether lethal mutations are generated to an equal extent by all fractionation schedules.

The efficacy with which lethal mutations were generated by single dose and fractionated irradiation schedules was compared by plotting the ratios of immediate:corrected survival against surviving fractions from conventional immediate plating (figure 7) using data from this work and from Chatterjee *et al.*

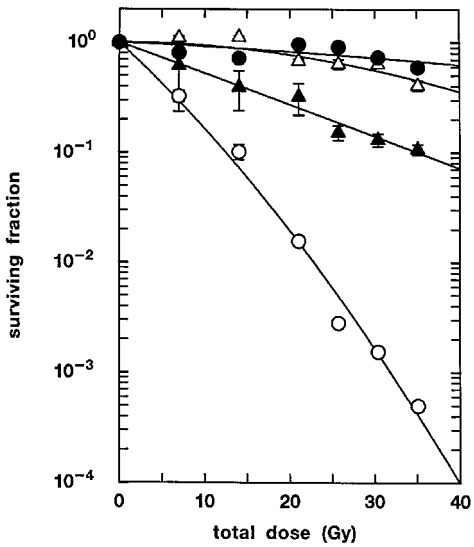

Figure 5. Survival of CaNT cells from tumours, treated *in vivo* with 3 daily radiation fractions of 2·34 Gy and propagated and replated at a range of times after irradiation, as a function of total dose and time of plating. (○) cells plated 6 h after irradiation, (▲) cells plated 13 days after irradiation, (△) cells plated 24 days after irradiation, (●) cells plated 35 days after irradiation. Error bars indicate standard errors of the mean and where not shown, are smaller than the size of the points plotted.

1995. A slope of $-0{\cdot}79 \pm 0{\cdot}12$ ($p=0{\cdot}0018$) was obtained for the single dose data, $-0{\cdot}61 \pm 0{\cdot}08$ ($p=0{\cdot}00014$) for two fractions per day and $-0{\cdot}41 \pm 0{\cdot}049$ ($p=0{\cdot}000008$) for three fractions per day. The slope fitted through the single dose data is biased by a single point and is not significantly different from the data for three fractions per day.

4. Discussion

It has been reported (Seymour and Mothersill 1986) that an increased death rate among the progeny of cells surviving acute radiation exposure persists for many generations after irradiation. The data presented here show that the plating efficiency of cells from tumours irradiated *in vivo* is reduced for many cell generations after irradiation. It is also evident that conventional survival data from cells plated soon after irradiation, considerably underestimate the amount of lethal damage inflicted on the cells. The reduction in long term viability of irradiated cell populations appears to be dose-dependant and is most noticeable after large doses of radiation, but is ameliorated to some extent by fractionation.

During the course of colony formation by irradiated survivors, non-viable cells are persistently produced during a proportion of the cell divisions. This has been interpreted as a sign of genetic instability or heritable LM, which lead to the production of non-viable progeny at some cell divisions. This phenomenon has been observed in several *in vitro* mammalian cell lines (Seymour *et al.* 1986, Mothersill and Seymour 1987, Gorgojo and Little 1989, Little *et al.* 1990, Chang and Little 1991). Significant cytogenetic instability is observed in the progeny of cells irradiated with alpha particles (Kadhim *et al.* 1992) and late expression of specific mutations and transformation following low LET radiation (Little *et al.* 1990, Mendonca *et al.* 1993). The potential persistence of cytogenetic instability in irradiated cells is of obvious concern and may provide a mechanism for the expression of LM.

It has been shown that the cells that can be grown from the CaNT tumour represent the aneuploid tumour cells and that the diploid stromal cells do not persist in culture, and are therefore not included in the data presented here. Microscopic observation of cultures suggested that the majority of lethally irradiated cells either never attach to the plastic dishes or detach after incubation. In addition, some may fail to survive trypsinization intact. This was confirmed by counting the number of cells in irradiated and control populations (Chatterjee *et al.* 1995). However, the potential problem of contamination of cultures by dead cells and by diploid stromal cells has been addressed in two ways.

First individual colonies were isolated, of normal size and appearance, and cells propagated from these colonies with further isolations from individual colonies after passaging (figures 2 and 3). Each colony is generally considered to be derived from a single viable cell. Therefore assessment of plating efficiencies of cells from normal sized colonies excluded all the original cells which were non clonogenic or had limited reproductive potential from the assay. Colonies isolated from excision assays of tumours irradiated with a large single dose *in vivo* had surprisingly heterogeneous plating efficiencies indicating that the original founding cell had produced progeny cells with little or no division potential in addition to a proportion of cells with sufficient division potential to form normal sized colonies and undergo further propagation. Heterogeneity in plating efficiency was observed between clonal lines within individual tumours and also between groups of cell lines isolated from different tumours. This may suggest that differences in the microenvironments within individual tumours at the time of irradiation may have had some influence on the subsequent delayed expression of the radiation damage.

Secondly cell sorting was used on the basis of DNA content to isolate G_1 aneuploid cells from a mixture of diploid stromal and aneuploid tumour

Table 2. Fraction of missing colonies and relative viability of descendants of survivor colonies after each passage following 1, 2 and 3-fraction irradiations *in vivo*.

Day/ no. fractions	Fraction no.	Total dose /Gy	Plating efficiency (%)	s.e.m.	Missing colony count*	Relative missing count*	Viable descendants of survivor colonies*
Day 0							
0	0	0	31.00	1.20	–	–	–
1	1	20.0	0.143	0.048	30.98	0.999	0.1
2	6	20.4	0.925	0.705	30.07	0.970	3.0
3	9	21.1	0.459	0.043	30.54	0.985	1.5
1	1	25.0	0.015	0.003	30.98	0.995	0.1
2	7	23.8	0.248	0.040	30.75	0.992	0.8
3	11	25.7	0.082	0.006	30.91	0.997	0.3
Day 12							
0	0	0	30.10	2.89	–	–	–
1	1	20.0	2.73	1.15	27.37	0.909	9.1
2	6	20.4	15.32	1.04	14.78	0.491	50.9
3	9	21.1	9.64	0.99	20.46	0.679	32.1
1	1	25.0	0.289	0.064	29.81	0.990	1.0
2	7	23.8	8.93	1.30	21.17	0.703	29.7
3	11	25.7	4.56	0.76	25.54	0.848	15.2
Day 24							
0	0	0	21.30	0.76	–	–	–
1	1	20.0	10.10	3.01	11.2	0.526	47.4
2	6	20.4	19.30	0.63	2.0	0.0939	90.6
3	9	21.1	17.27	0.52	4.03	0.189	81.1
1	1	25.0	5.03	2.51	16.27	0.764	23.6
2	7	23.8	19.95	0.84	1.35	0.063	93.7
3	11	25.7	16.24	1.55	5.06	0.238	76.2
Day 35							
0	0	0	20.30	1.36	–	–	–
1	1	20.0	14.20	1.50	6.1	0.300	70.0
2	6	20.4	18.50	0.94	1.8	0.089	91.1
3	9	21.1	23.16	0.97	0	0	100.0
1	1	25.0	9.74	2.42	10.56	0.520	48.0
2	7	23.8	16.92	0.89	3.38	0.166	83.4
3	11	25.7	21.95	1.49	0	0	100.0

*Per 100 cells.

cells, disaggregated from tumours irradiated *in vivo*, (figure 4B) and they were plated without feeder cells. This experiment, by excluding the normal stromal population of the tumour and potential residual feeder cells from the survival assays, demonstrated the generation of lethal mutations within the aneuploid population.

Seymour *et al.* 1986 corrected conventional survival data (figure 5) by multiplying them by data obtained from repetitive replating of the cells after subsequent passages (figure 6). The ratio of the conventional: corrected survival data (equation (3)) is a measure of the persistence of reduced viability of cell progeny in the later re-platings. If all the survivors from each dose group and radiation schedule had similar plating efficiencies after passaging to those of untreated cells, then allowing for statistical noise in the assay, all ratios of immediate: corrected survival would be expected to cluster around a value of 1·0. Comparing data from this work and from Chatterjee *et al.* 1995 (figure 7) there is a clear trend for higher ratios of immediate: corrected survival to be obtained as the conventional surviving fraction falls with increasing severity of treatment. There is also a non-significant trend for higher ratios from single (solid line) compared with the pooled fractionated irradiations (dashed line) with the higher doses. Similar ratios of conventional: corrected survival were observed from the two and three fractions per day schedules. In both schedules, the minimum interfraction interval was sufficient for complete repair of reparable damage to occur. However, the influence of fractionation, and therefore damage repair on the delayed expression of lethal damage implies that the extent of this component of damage may depend on the cellular repair proficiency.

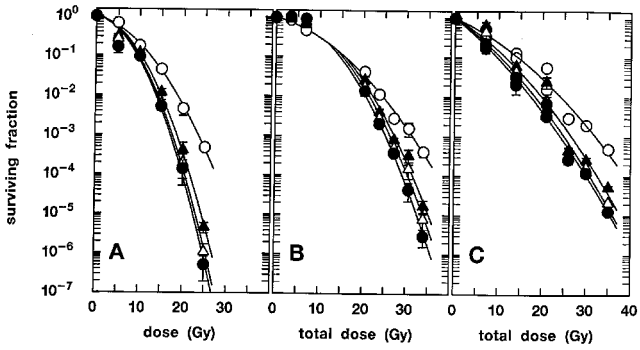

Figure 6. Comparison of data from CaNT tumours, treated *in vivo* with single doses or up to 10–15 fractions over 5 days as a function of total dose and time of plating, corrected for the reduced viability of progeny. Data for tumours treated with single radiation doses (A), 2 daily radiation fractions (3·4 Gy) (B), three daily radiation fractions (2·34 Gy) (C). Data in (A) and (B) were reported in previous work (Chatterjee *et al.* 1995). (○) cells plated 6 h after irradiation, (▲) cells plated 13 days after irradiation, (△) cells plated 24 days after irradiation, (●) cells plated 35 days after irradiation. Error bars represent standard errors of the mean and where not shown are smaller than the size of the points plotted.

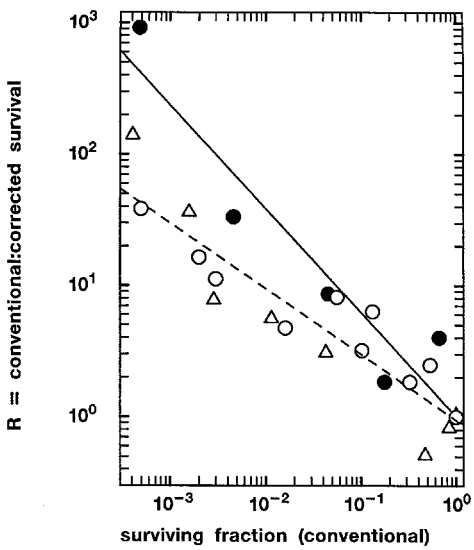

Figure 7. Ratios of immediate:corrected survival versus immediate survival from irradiation with 20–35 Gy delivered as (●) single doses, (△) 2 fractions (3·4 Gy) per day or (○) 3 fractions (2·34 Gy) per day over 5 days. The solid and dashed lines were fitted, through the logarithmically transformed single dose and pooled fractionated data respectively, to the equation $R = 1/k \times SF_0^P$ using the method of least squares.

Most *in vitro* predictive assays of intrinsic cellular sensitivity to radiotherapy and other cancer treatment modalities (Courtenay *et al.* 1978, Fertil and Malaise 1981) rely on a single clonogenic or other determination of cell survival, a short time after treatment, and will not be informative about the long-term changes in the proliferative potential of the cells. Moreover, by ignoring the reduced viability of the cells surviving each radiation fraction predictive assays used to measure the surviving fraction at 2 Gy underestimate the amount of damage done to the cells, and may compromise attempts at carrying out more sophisticated predictive modelling of therapeutic regimes. In the context of predictive assays for radiosensitivity, it is of particular concern that this form of damage may depend on repair proficiency and may therefore be expressed differently in samples from different individuals.

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