Pergamon

European Journal of Cancer, Vol. 35, No. 5, pp. 850–858, 1999

Printed in Great Britain
0959-8049/99/$ - see front matter

PII: S0959-8049(99)00019-2

Original Paper

Cell Kinetics and Repopulation Parameters of Irradiated Xenograft Tumours in SCID Mice: Comparison of Two Dose-fractionation Regimens

E. Sham[1] and R.E. Durand[2]

[1]British Columbia Cancer Agency, Department of Radiation Oncology, Vancouver; and [2]British Columbia Cancer Research Centre, Department of Medical Biophysics, 601 West 10th Avenue, Vancouver, British Columbia, V5Z 1L3, Canada

The extent and mechanism(s) of repopulation were assessed in SiHa (human cervical squamous cell carcinoma) xenografts in SCID mice for two fractionated irradiation regimens. Mice in one arm of the study received 50 Gy in 20 fractions over 23 days with a 14 day split between 10 fraction, 5 day courses. The other tumours were treated with 50 Gy in 20 fractions over 10 consecutive days. Cell kinetics and tumour regrowth parameters were monitored during and after treatment by measuring tumour volume and analysing cellular DNA content and proliferation parameters with flow cytometry. Repopulation occurred rapidly, beginning during irradiation and largely attributable to an increased growth fraction and decreased potential doubling time, apparently triggered by increased cell loss. Cell cycle time, in contrast, remained relatively constant throughout. Extrapolation of these results to humans suggests that treatment times should be minimised whenever possible, since regrowth rates exceeded those predicted from pretreatment T_{pot} measurements. © **1999 Elsevier Science Ltd. All rights reserved.**

Key words: radiotherapy, accelerated repopulation, cell kinetics, T_{pot}, xenografts, predictive assays
Eur J Cancer, Vol. 35, No. 5, pp. 850–858, 1999

INTRODUCTION

THERE IS now an overwhelming body of clinical and experimental evidence that radiotherapy is subject to a treatment time effect, such that for a given prescribed dose, there is a decreased likelihood of controlling the tumour as the overall treatment duration increases [1–9]. The clinical significance of the time effect, however, appears to vary with tumour site. None the less, the unifying concept of 'accelerated repopulation' has emerged to explain these observations [1].

Since rapid tumour recurrences after radiotherapy are not uncommon, it would seem that those tumours might be particularly useful for understanding regrowth properties. Unfortunately, rapid recurrences alone do not indicate when (or even if) accelerated growth might have begun. Repopulation remains implicated as a major determinant of the differences in outcome however, as there typically have been few demonstrable effects of fraction size in the clinical series assessed for accelerated repopulation [5, 8–11]. This is an important question, because failure due to inherent resistance leaves fewer options for modifying treatment than does the recognition of repopulation during radiotherapy.

Two complementary yet distinct issues are the duration of the lag period between the start of radiotherapy and commencement of accelerated tumour (re)growth, and the actual mechanism(s) of 'acceleration'. There clearly would not be any kinetic benefit to accelerating a regimen that was already shorter than the regrowth lag time, so it is of obvious importance to have a precise estimate of that time. Unfortunately, that is seldom the case; the most extensive clinical data from retrospective analyses of head and neck cancer suggest a broad interval from 17 to 33 days [8, 12]. While arguably of less practical importance, the issue of the actual mechanism(s) of acceleration is similarly unresolved [13]. The debate continues on whether 'acceleration' is simply a result of cell loss and changing growth fraction in the tumour [14], or

Correspondence to R.E. Durand, e-mail: rdurand@bccancer.bc.ca
Received 30 Jul. 1998; revised 2 Dec. 1998; accepted 15 Dec. 1998.

whether cell cycle changes similar to those in repopulating normal tissues occur [15]. Irrespective of the mechanism, tumour repopulation remains a clinical problem.

Treatment interruptions are inevitable; public holidays and machine breakdowns occur frequently. Short breaks of 3 days, such as over the Christmas period, may result in a 5% decrease in local control probability for some tumours. It would be desirable to compensate for these interruptions, yet not all tumour sites appear to be significantly affected by radiotherapy treatment time [7, 16, 17]. In which tumours should one compensate for treatment interruption?

It seems obvious that for intrinsically fast growing tumours, and those that show an early acceleration of growth during therapy, prolonged treatment will adversely affect local control. Unfortunately, the hope that potential tumour doubling time, T_{pot}, would identify tumours with rapid repopulation potential has not been realised [18–20]. Tumours with short values of T_{pot} are clearly problematic, but a longer T_{pot} can result from either a slow inherent growth rate or a low pretreatment growth fraction; only the former guarantees slow regrowth. An assay that could reliably identify tumours with high growth potential, and especially those capable of rapid repopulation even during therapy, would, therefore, help radiation oncologists in selecting an optimal treatment schedule. Surprisingly, developing such an assay has not received a high priority.

We have addressed the question of identifying tumours with rapid potential growth rates, and those that exhibit repopulation early in treatment, by developing flow cytometry-based assays to measure the cell cycle time, t_c, with a single biopsy before or during therapy [21, 22]. When the biopsy is performed during therapy, both an indication of the fraction of clonogenic cells (a measure of the inherent radiosensitivity of the tumour) and the cycle time of those clonogenic cells (a measure of repopulation potential) can be forthcoming. In this paper, we report the results of experiments designed to test the utility and accuracy of these techniques in dose-fractionation schedules expected to be at the extremes of regrowth permissiveness. Repopulation was concomitantly assessed by independent criteria.

We have continued to use the SiHa human cervical squamous cell carcinoma line grown as xenografts in SCID mice and irradiated with 2.5 Gy fractions twice daily with a 7 h interfraction interval. Data are reported here for 50 Gy delivered in 10 consecutive days versus the same dose delivered as 2×25 Gy/5 days with a 14 day interval. In addition to the assessment of our analytical techniques in each regimen, this design also allowed intercomparisons with previous data [22] to assess reproducibility of the assays and their predictivity.

MATERIALS AND METHODS

Experimental design

The established, transplantable SiHa human tumour cell line [23] was obtained from ATCC (Rockville, Maryland, U.S.A.) then maintained as an intramuscular transplant in SCID mice. Experimental tumours were grown as concurrent subcutaneous implants on both the back and flank; one million cells (0.05 ml of 2×10^7 cells/ml suspension) were inoculated subcutaneously at each site. All animals were housed in microisolators with water and food provided *ad libitum*. Tumours were used for experiments approximately 4 weeks after implantation (200–300 mg in weight).

Irradiation

Two experimental irradiation protocols were used. In arm 1, tumours were irradiated to 50 Gy in 20 fractions with 250 kVp X-rays at a dose rate of 0.4 Gy/min as a split course over 23 days. The initial 25 Gy of radiation was delivered in 10 treatments over 5 days (designated days 0–4), with a daily interfraction interval of 7 h. The final 25 Gy was given in the same manner (days 19–23) after a treatment break of 2 weeks. Mice in arm 2 were treated to 50 Gy in 20 fractions over 10 days continuously (days 0–9). Irradiation was confined to the back tumours, with the mice immobilised in specially designed jigs that incorporated lead shielding around vital organs and over the flank tumours. X-rays were delivered using parallel-opposed fields to ensure a more uniform dose distribution.

Data collection and analysis

Tumour volumes were measured twice weekly before irradiation (days − 8–0), then once every 2 days during treatment and the subsequent observation period (days 0–29). The clonogenic fraction *in vitro* and flow cytometry-based cell kinetics parameters were measured each treatment day and on alternate days when not on treatment. The length, width and height of each tumour were measured by the same investigator throughout the experiment and tumour volumes were calculated as the product of the three dimensions multiplied by $\pi/6$ (a hemi-ellipse). Relative tumour volume (RV) was defined as the ratio of the average tumour volume on a given day of the experiment to the average tumour volume immediately before the first dose of radiation. Volume doubling time (t_d) was the time required for the measured tumour volume to increase 2-fold. Growth delay was defined as the time required by the treated tumour to attain a volume twice as large as the volume at the start of treatment minus the corresponding time for control tumours to achieve the same 2-fold size increase.

Pairs of mice were randomly selected from each study arm for DNA labelling 1 h after the first daily treatment during the irradiation period (receiving no further radiation treatments) and at the same time on alternate days during the post-treatment observation interval. These mice were injected intraperitoneally with iododeoxyuridine (IdUrd, 90 mg/kg from a 6 mg/ml stock solution in 1.0 mM Tris base, pH 10). After 24 h, each of these mice was injected intraperitoneally with bromodeoxyuridine (BrdUrd, 90 mg/kg from a 6 mg/ml stock solution in phosphate-buffered saline (PBS)). Four hours later, tumours were excised, then finely minced and agitated in an enzyme suspension containing 0.5% trypsin and 0.08% collagenase for 40 min before adding 0.06% DNAase. The cell suspension was then gently vortexed and filtered through 30 μm Nylon mesh to remove clumps and the monodispersed cells were washed by centrifugation. An appropriate portion of the cell suspension was saved for clonogenicity measurement and the remainder was fixed in chilled 70% ethanol. For clonogenicity, cells were plated in Eagle's minimal essential medium supplemented with 10% fetal calf serum and antibiotics. Colonies were stained and counted 2 weeks later; data have been expressed as the observed clonogenic fraction of the cells recovered and as the estimated number of remaining clonogenic cells per tumour site (the product of the total cells recovered per tumour and the clonogenic fraction of those cells).

The two monoclonal antibodies (MAb) selected for the double labelling procedure were Br-3 (anti-BrdUrd, Caltag

Laboratories, San Francisco, California, U.S.A.) at a 1:100 dilution and FITC (fluorescein isothiocyanate)-conjugated B-44 (anti-IdUrd and anti-BrdUrd, Becton Dickinson Immunocytometry Systems, San Jose, California, U.S.A.) at a 1:50 dilution. An IgG-Cy3 goat antimouse antibody (Caltag Laboratories) at a 1:50 dilution was used as the secondary antibody against Br-3. Applying the Br-3 antibody (and secondary) first masked the BrdUrd binding sites for B-44; additionally, B-44 preferentially stains IdUrd under the conditions used, therefore, allowing quite good specificity. Finally, DNA was stained with DAPI (4,6-diamidino-2-phenylindole dihydrochloride hydrate) at 1 μg/ml. List mode files were collected using a dual laser Epics Elite-ESP flow cytometer (Coulter Corporation, Hialeah, Florida, U.S.A.) and subsequently reprocessed for analysis. Doublet correction and bitmap gating were used to select the tumour cell populations with the WINLIST software package and univariate DNA histograms were analysed with the MODFIT package (both from Verity Software House, Topsham, Maine, U.S.A.).

Based on whether the IdUrd-labelled cells had completed more or less than a full cell cycle at the time of analysis, supplemented with information on the rate of cell progression (from the BrdUrd-labelled cells), the cell cycle time t_c was estimated [21, 22]. In essence, t_c is the usual intermitotic time. Functionally, however, the measurement is the time required for early S phase cells to progress to the same point in the subsequent S phase and, therefore, reflects only those cells that undergo consecutive rounds of DNA synthesis within the defined observation time. Our reported labelling indices (LI) were corrected for cell progression; for the BrdUrd-labelled cells, both the numerator (labelled cells) and the denominator (total cells) were decreased by half the number of labelled G_1 cells. For IdUrd, all labelled G_1 and S phase cells were similarly assumed to have divided after labelling. Potential doubling time (T_{pot}) was determined cytometrically for the BrdUrd-labelled cells and was proportional to the ratio of the duration of S phase (t_s) and LI. All t_s values were estimated using both the conventional 'relative movement' approach [24, 25] and the S phase exit (%S) method [26]; very similar results were obtained and all data reported here were from the latter method. Standard definitions of growth fraction (GF, the ratio of t_c to T_{pot}) and cell loss factor ($\Phi = 1 - T_{pot}/t_d$) were also employed [21, 27].

RESULTS

Growth of untreated SiHa tumour xenografts, expressed as RV, increased non-exponentially as expected. The growth rate decreased as the tumours aged; tumour volume doubling time (t_d) was 6 days on day 0, increased to 10 days on day 10 and to 14 days on day 20 (Figure 1). In irradiated tumours, tumour volume was an insensitive indicator of response. The RV increased less rapidly after commencement of irradiation, an effect dependent on treatment intensity. At day 4 of treatment, tumours began to regress and further growth in the split course regimen only became obvious after approximately another week (Figure 1; the curves for treated tumours have been drawn in black during the treatment intervals and broken at other times). Once regrowth was established, the growth rate was quite comparable to control tumours of the same size (at the commencement of the second part of the split course, day 19, t_d was not significantly different from the pretreatment value). Overall, an apparent growth delay of 11 days resulted from the first treatments in the split course regimen. During and after the second part of the split course treatment, RV changed as during the first irradiations.

The RV and t_d of tumours treated with the continuous course of radiation naturally were indistinguishable from those in the split course during days 0–4. With continued therapy, however, the continuously treated tumours further decreased in size. They continued shrinking until the end of the observation period, at which time the RV was 0.3. Due to the radiosensitivity of the exposed normal tissues of the SCID animals, the post-treatment observation period after the more intensive treatment was not extended to allow regrowth.

The clonogenic fraction of cells from the control tumours averaged 21% and cell recoveries were typically 5×10^7/g. As illustrated in Figure 2a, the clonogenic fraction decreased rapidly during irradiation. Nearly a three-decade decrease in relative clonogenicity was measured on day 4 in arm 1, i.e. after 25 Gy. Recovery, however, occurred rapidly during the treatment break and the clonogenic fraction returned to the pretreatment value by day 15 (note that this observation conclusively establishes that all radiation-induced dead and dying cells had disappeared from the tumour, but does not necessarily imply that pretreatment *numbers* of clonogenic cells were present). The fraction of clonogenic tumour cells decreased rapidly again when treatment was re-initiated on day 19 and remained low until the end of observation.

The fraction of clonogenic cells, as a single parameter, was less meaningful in the more intensive continuous course, due to concomitant cell loss. Five to six decades of cell kill were observed in some tumours, but most showed an apparent

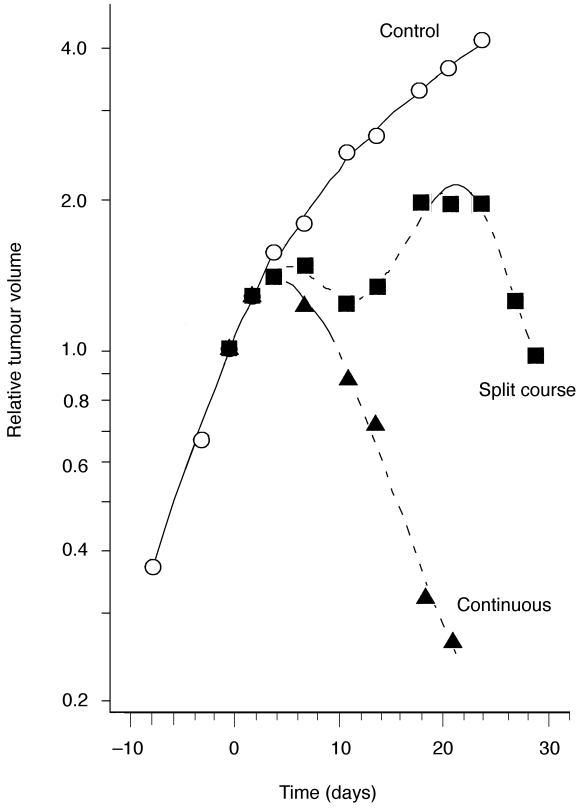

Figure 1. Relative tumour volume (RV) as a function of time. ○, untreated tumours; ■, split course irradiation; ▲, continuous course. Broken lines show tumour response when not on treatment.

increase in clonogenic fraction despite continued irradiations after day 7. This clearly suggests a selective loss of non-clonogenic cells; the cells remaining in the tumours tended to be those still clonogenic. We therefore normalised the results for both changes in tumour size and in cell recovery and, thus, estimated the time-dependent number of clonogenic cells remaining at the tumour site (Figure 2b).

Several features of this analysis were unexpected. First, although the continuous treatment did produce a further reduction in clonogens, the last 5 days of treatment were considerably less effective than the first. Given the rapid repopulation seen in the split course regimen and following the continuous exposures, clonogen repopulation must have begun early in the treatment and by the later stages of the continuous arm was proceeding at a rate nearly sufficient to balance the radiation-induced cell kill. Second, the comparable rates of apparent 'regrowth' in Figure 2a, in contrast to the slower repopulation of clonogens in Figure 2b, suggest that loss of clonogenic cells may also have occurred during both the shrinkage and regrowth phases. A last point is the speed of the clonogen repopulation. Even without loss of clonogens, restoration of four decades of cells would require more than 13 cell divisions ($2^{13} \approx 10^4$). For this to occur in 14 days, the cycle time t_c of the clonogenic cells must be of the order of 1 day, much faster than the tumour doubling time (t_d) of 6 days, or even the pretreatment potential doubling time (T_{pot}) shown in Figure 3.

Actual measurements of t_c based on flow cytometry techniques with two halogenated pyrimidines [21, 22] showed relatively constant values of 26 ± 2 h in both control and irradiated tumours (Figure 3a). Conversely, in both control and irradiated tumours, T_{pot} was more time dependent. In controls, T_{pot} was initially somewhat less than 100 h and as expected, increased with time. In the irradiated tumours, T_{pot} was initially higher than for controls (presumably a combination of radiation-induced changes in LI and proliferating fraction), but decreased during each treatment course. Interestingly, T_{pot} never shortened to values approaching t_c; also, T_{pot} lengthened as repopulation progressed.

LI averaged 12% in the control tumours (Figure 4). Both irradiated groups had a slight reduction in LI after the first exposure, consistent with the increased T_{pot} in the previous figure. Subsequently, at the times of BrdUrd administration in the irradiated tumours, i.e. 25 h after the last radiation exposure, LI increased during each course of radiotherapy. In the split course regimen, LI peaked at 20% at day 7 and had returned to the pretreatment value at day 18. It then increased again with the second series of irradiations, exceeding 30% at day 28. With continuous fractionation, LI increased to 30% at day 9. Interestingly, the rate of increase of LI was quite consistent through each series of radiation treatments.

The pretreatment GF (t_c/T_{pot}) was calculated to be 24% (Figure 5). Overall, GF changes were proportional to those of LI. GF increased in the split course arm to 60% at day 8, then returned to the pretreatment value at day 18, just before commencing the last 10 fractionated irradiations. Similar

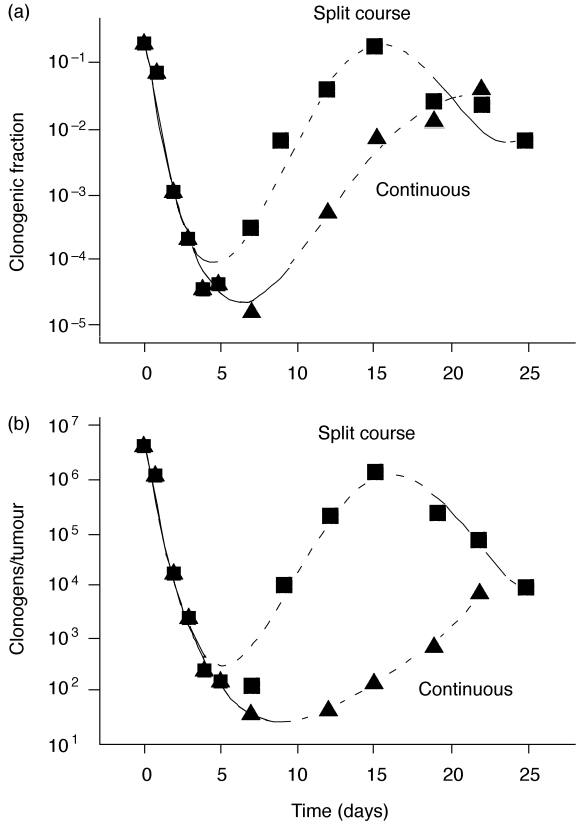

Figure 2. Clonogenic fractions of recovered cells (a) and the number of clonogenic tumour cells remaining (b) after correction for the changes in tumour size and cell recovery. Note the rapid repopulation after the first course of irradiation in the split course group. ■, split course irradiation; ▲, continuous course. Broken lines show tumour response when not on treatment.

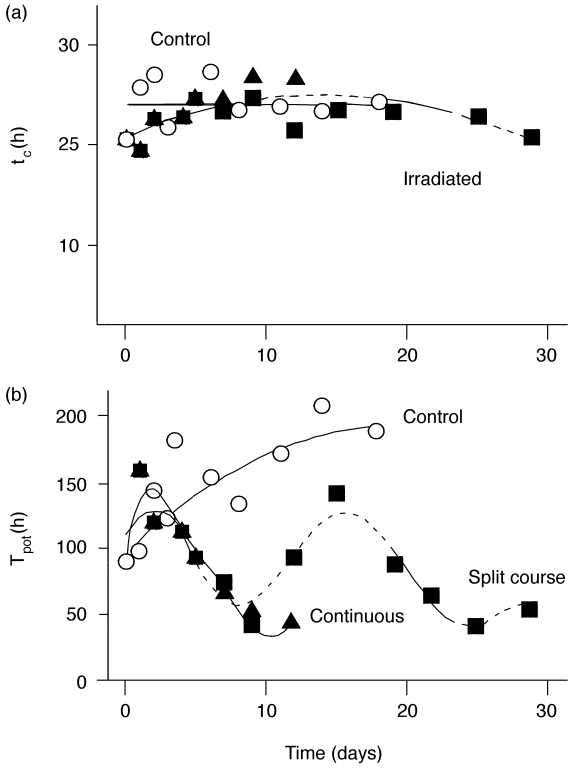

Figure 3. (a) The cell cycle time (t_c) and (b) potential doubling time (T_{pot}) of control and irradiated SiHa xenografts after initiating treatment. Note that t_c was relatively constant and correlated better with regrowth rate than did T_{pot}. ○, untreated tumours; ■, split course irradiation; ▲, continuous course. Broken lines show tumour response when not on treatment.

changes were seen during the second course of irradiation in this group and in tumours of the continuous course group, where the GF exceeded 60% on day 8 and remained high until the end of observation.

Cell loss was not unlike GF. The cell loss factor (Φ) of control tumours was approximately 0.5. This increased to the maximal value of 1.0 at day 6 in arm 1, but unlike most other parameters, did not return completely to pretreatment values during the treatment break. A value of 0.7 was seen at the start of the later irradiations and Φ again reached 1.0 by the end of the radiation treatments. In arm 2, Φ approached 1.0 by day 6 and showed no sign of decrease throughout the observation interval. Interestingly, Φ remained high even when the tumour cells were repopulating maximally and T_{pot} had reached its nadir value.

In control tumours, G_1, S and G_2 populations averaged 62%, 21% and 17%, respectively, based on mathematical analyses of single parameter DNA flow histograms and assuming a negligible fraction of higher ploidy cells. A radiation-induced G_2 block was observed in both treatment arms and the cell cycle perturbations produced in the split course regimen are shown in detail in Figure 6. The G_2 block, produced by each series of radiation treatments, was offset by a compensatory decrease in the G_1 population. The initial block resolved promptly after day 4 and by day 15 the DNA histograms were indistinguishable from those obtained pretreatment. The G_2 block produced during the second course of therapy after the treatment break showed similar induction and resolution kinetics. Interestingly, the proportion of S phase cells increased during and after treatment, presumably demonstrating the recruitment of quiescent (G_0) cells into the active cell cycle during irradiation.

Unexpectedly, the G_2 block resulting from continuous radiotherapy did not increase monotonically throughout treatment at the expense of G_1 cells. Rather, towards the completion of treatment, the G_2 cells appeared to diminish in relative number, with a concurrent increase in G_1 and S phases. This observation, which suggests active repopulation even during the continuing radiotherapy, is also illustrated by the sequential DNA distributions in Figure 7 where concomitant BrdUrd uptake provides an indication of both viability and progression. Due to the loss of dead cells, the proportion of BrdUrd-incorporating cells (putative clonogens) among the recovered cells increased progressively after day 7. Since the hypoxic fraction of SiHa xenografts is of the order of 40% and largely consists of G_0/G_1 cells, it is interesting to note that in these experiments (as in previous ones [28]), hypoxic cells were apparently recruited into the proliferating compartment early in the treatment regimen, in turn implying quite rapid reoxygenation.

DISCUSSION

Cell kinetics and repopulation parameters during regimens delivering a relatively high-dose of 50 Gy as a continuous or split course schedule have been evaluated here in SiHa tumour xenografts. Since our long-term goal is to develop an *in situ* predictive assay that encompasses both inherent radiosensitivity and regrowth potential, the fact that the flow cytometry measurements made throughout these regimens were both internally consistent and very comparable to those reported in an earlier preliminary study [22] suggests that this approach may have clinical potential.

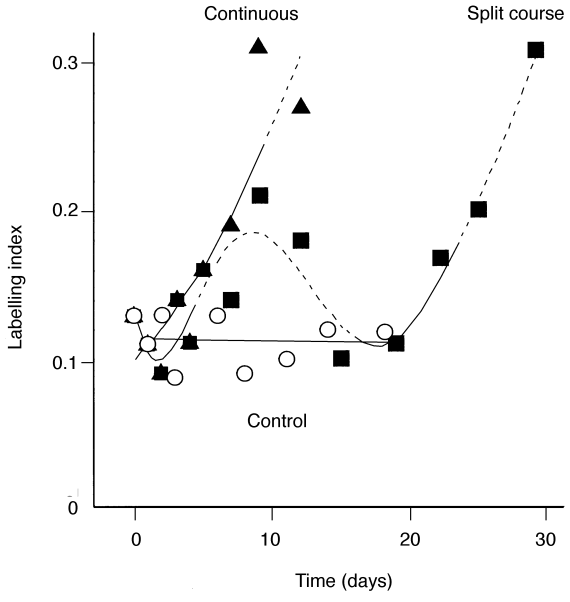

Figure 4. Labelling index (LI) for bromodeoxyuridine (BrdUrd) uptake. The number of labelled cells increased markedly during and after each course of multifraction irradiation, but also returned to the pretreatment value prior to commencing the second course of therapy in the split course group. ○, untreated tumours; ■, split course irradiation; ▲, continuous course. Broken lines show tumour response when not on treatment.

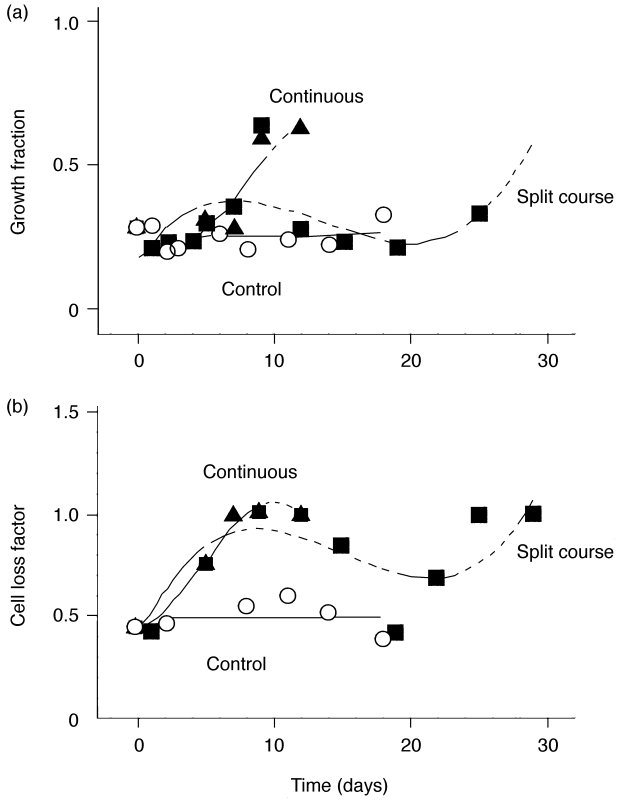

Figure 5. Changes in (a) growth fraction (GF) and (b) cell loss (Φ) derived from data in previous figures. Irradiation rapidly increased GF, offset to an extent by a sustained increase in Φ (plotted at 1.0 when tumours were regressing). ○, untreated tumours; ■, split course irradiation; ▲, continuous course. Broken lines show tumour response when not on treatment.

The fractionation scheme of 50 Gy in twice-daily 2.5 Gy fractions was clearly a compromise, adopted to be as relevant as possible to a radical course of radiation treatment in the clinic, yet feasible in the xenograft model. Split course irradiation is not uncommon for radical, adjuvant and palliative treatments, and is often combined with chemotherapy for non-palliative intents. Clinically, the rest period allows the patient to recover from the acute reactions before the second portion of radiation is delivered; in these studies, it provided both the opportunity to identify and evaluate early regrowth and later to assess the functionality of our flow cytometry techniques in a pre-irradiated tumour.

Even in model systems, a major uncertainty is introduced by the fact that the number of viable cells in a given tumour cannot be measured directly (recall Figure 2); increases in the clonogenic fraction, for example, can reflect increasing numbers of viable cells and/or decreasing numbers of non-viable cells. Even the estimates of clonogens per tumour reported here are subject to the caveats that clonogenicity was measured *in vitro* not *in situ* and that it was implicitly assumed that all clonogens were *always* recovered. Nevertheless, repopulation throughout treatment can be safely inferred, since some cells incorporated the halogenated pyrimidines and progressed, GF and LI increased, T_{pot} decreased and restoration of the pretreatment numbers of clonogens was much more rapid than expected from pretreatment tumour kinetics. Repopulation during the treatment break or after completion of the treatment was less ambiguous, as the increasing clonogenic fraction of tumour cells was coincident with increases in both tumour volume and tumour cell recovery (assuming, of course, that the probability of recovering any particular clonogen remained constant throughout this period). Overall, while there can be little doubt that quite rapid regrowth was observed, it is worth noting that both

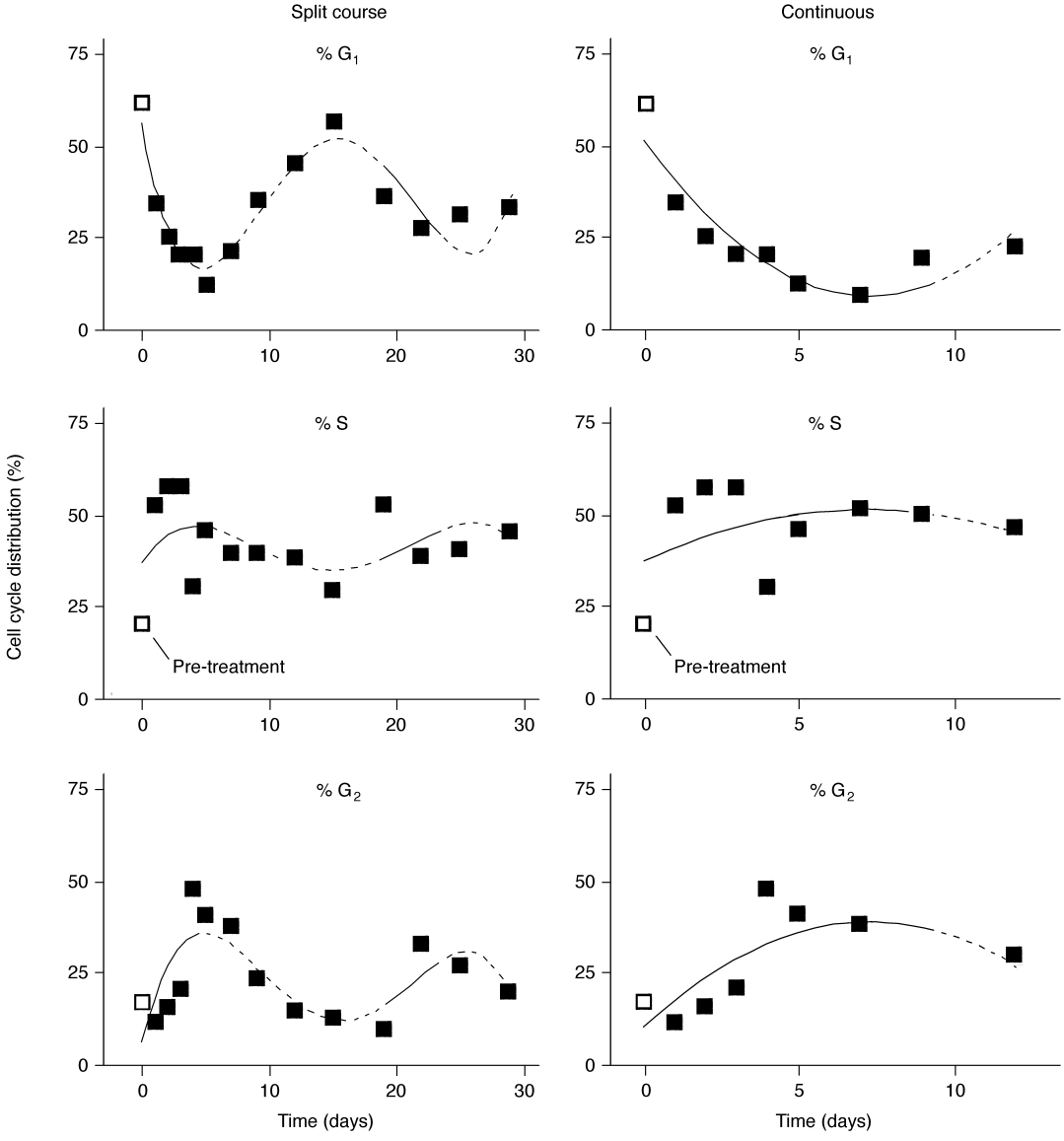

Figure 6. Cell cycle distribution changes in tumours treated by split course or continuous course irradiation. A G_2 block occurred during each course of therapy, then promptly resolved. With continuous course irradiation, the progressive accumulation of G_2 cells initially seen decreased with time and the corresponding increase of S phase cells was suggestive of repopulation even during treatment. Note, however, that viable and non-viable cells cannot be distinguished in these analyses. Broken lines show tumour response when not on treatment.

'tumour volume' and 'clonogenic fraction' are potentially ambiguous measures of regrowth: for the split course regimen, Figure 2a suggests a return to 100% clonogenic cells by day 12 and from Figure 1, the tumour size peaked at nearly double the pretreatment size. However, from Figure 2b, the number of clonogenic cells per tumour always remained at least 10-fold less than the starting value, suggesting that the rate of increase of tumour clonogens, a more rigorous measure of repopulation, was less rapid.

T_{pot} diminished rapidly after commencement of treatment, but did not reach a minimum value particularly close to the t_c level. Pre- and intertreatment T_{pot} estimates, therefore, both underestimate the regrowth potential of these tumours, an observation consistent with the emerging consensus that T_{pot} is not always useful in the clinic [18–20, 22, 29]. It is of particular importance that of the kinetic parameters measured in these studies, only t_c was invariant; each of the others (T_{pot}, RV, t_d, CF, LI, GF and Φ) was critically dependent upon the time of measurement, and notably, the treatment intensity.

In both the split course and continuous regimens, the DNA distributions of the SiHa tumour cells showed rapid and dose-responsive changes during treatment. It was of particular interest that a G_2 block appeared and that the G_1 cells decreased by a value considerably larger than that pretreatment fraction of S phase cells. This in turn suggests that many of the normally quiescent (and often hypoxic) G_0/G_1 cells rapidly re-entered the proliferating cell compartment during therapy. It appears to be largely a semantic issue as to whether accelerated repopulation was seen. Clearly, the clonogenic cells increased in number at a more rapid rate than in the pretreatment tumours, so using that as the standard of comparison, then 'acceleration' was observed. However, the cell cycle time, t_c of the irradiated tumour cells was never faster than that of the untreated tumours, so the actual intermitotic time did *not* accelerate. Consequently, our data show that the rate of expansion of the clonogenic subpopulation did indeed increase, but only due to an increasing GF and decreased loss of clonogenic cells.

Clearly, the multifactorial nature of tumour response to radiation makes it very difficult to intercompare these results with others, as does the current experimental design which focused on the questions of whether and when clonogen repopulation occurs during radiotherapy. Other tumours with different growth controls and kinetics, different sensitivity to radiation-induced cell killing and cell cycle perturbations and different cell loss rates and mechanisms would naturally be expected to respond differently in time and degree. None the less, the demonstration that human tumour cell clonogens can continue to proliferate and even expand *during* radiotherapy suggests that reliable measurements of the cell cycle time of those clonogens may be of greater practical use than T_{pot} in the clinic. Support for that position is similarly forthcoming from several other experimental studies in which pretreatment T_{pot} was not found to be indicative of tumour growth potential or rate [22, 30–33].

A related, and important, question is the reoxygenation rate during therapy. We infer that these tumours reoxygenated fairly rapidly; since the SiHa xenografts typically show a hypoxic fraction of approximately 40% that is almost entirely G_0/G_1 cells, the rapid exit of cells out of G_1 during treatment implies an improvement in oxygenation and nutrition. We had hoped that our consecutive measurements of clonogenicity might provide an indication of changing radiosensitivity, but the combined problems of tumour to tumour variability, uncertainties in cell recovery and a lack of sensitivity at the low doses per fraction were all confounding

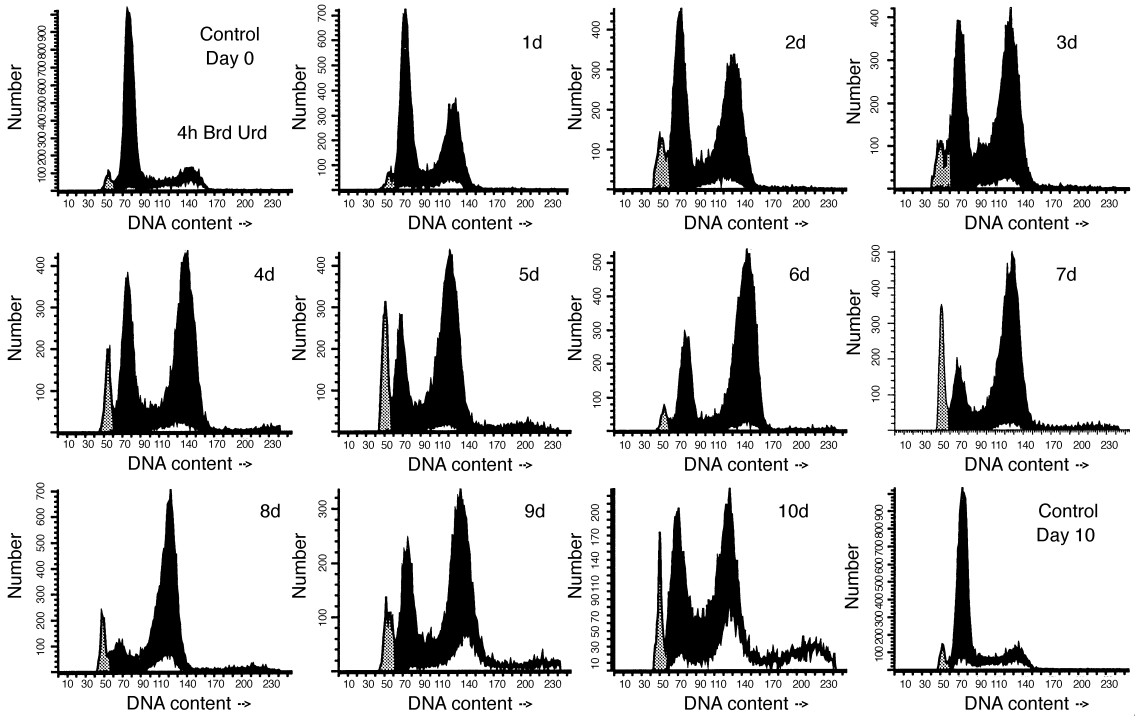

Figure 7. DNA distributions for tumour cells (black), normal cells (grey) and the bromodeoxyuridine (BrdUrd)-labelled subset (white) in tumours treated with continuous course irradiation. Rapid clonogen repopulation late in therapy is indicated by the increase in labelled cells and reversal of the G_2 block after 8 days.

factors that ultimately prohibited definitive measurements of changing radiosensitivity. The role of reoxygenation and particularly reoxygenation *rate*, thus requires further evaluation.

Although our experimental design, using different fractionation regimens with a single tumour system, can only indirectly address the question of whether our techniques will ultimately predict tumour responsiveness, we none the less find the data very encouraging. For purposes of illustration, consider our data 10 days after initiating treatment in each regimen. The conventional endpoint of tumour volume obviously provided little discrimination at that point, despite the three-decade difference in clonogenic cells per tumour. Similarly, none of the flow measurements, taken alone, showed an obvious difference. However, the combination of the reduced cell yield, perturbed cell cycle distributions and the large fraction of rapidly proliferating cells in the arm 2 tumours, together indicate that considerable depopulation had occurred, but that repopulation was ongoing. This is, we believe, exactly the type of information needed if a therapeutic regimen is to be altered: the tumour is radiosensitive, but sufficiently capable of repopulation that treatment acceleration is indicated.

In conclusion, our standard cytometric analyses appeared to be reliable and predictive in SiHa xenografts. Additionally, repopulation of clonogens was demonstrated even during treatment; the progressively increasing rate of clonogen expansion was not an accelerated growth rate *per se*, but rather, increasing numbers of proliferating cells. While we would not expect quantitatively similar results for human tumours that have markedly lower GFs and are treated with typical clinical regimens, we believe that qualitatively similar principles should none the less apply. The ability to make meaningful measurements during fractionated therapy regimens and particularly during high-intensity treatment courses, increases our optimism that techniques similar to those described here may eventually be tested in the clinic, and hopefully, find utility.

1. Withers HR, Taylor JM, Maciejewski B. The hazard of accelerated tumor clonogen repopulation during radiotherapy. *Acta Oncol* 1988, **27**, 131–146.
2. Allam A, Perez LA, Huang P, *et al.* The effect of the overall treatment time of fractionated irradiation on the tumor control probability of a human soft tissue sarcoma xenograft in nude mice. *Int J Radiat Oncol Biol Phys* 1995, **32**, 105–111.
3. Bentzen SM, Thames HD. Overall treatment time and tumor control dose for head and neck tumors: the dog leg revisited. *Radiother Oncol* 1992, **25**, 143–144.
4. Duncan W, MacDougall RH, Kerr GR, Downing D. Adverse effect of treatment gaps in the outcome of radiotherapy for laryngeal cancer. *Radiother Oncol* 1996, **41**, 203–207.
5. Fowler JF, Lindstrom MJ. Loss of local control with prolongation in radiotherapy. *Int J Radiat Oncol Biol Phys* 1992, **23**, 457–467.
6. Fyles AW, Keane TJ, Barton M, Simm J. The effect of treatment duration in the local control of cervix cancer. *Radiother Oncol* 1992, **25**, 273–279.
7. Hansen O, Overgaard J, Hansen HS, *et al.* Importance of overall treatment time for the outcome of radiotherapy of advanced head and neck carcinoma: dependency on tumor differentiation. *Radiother Oncol* 1997, **43**, 47–51.
8. Peters LJ, Withers HR. Applying radiobiological principles to combined modality treatment of head and neck cancer—the time factor. *Int J Radiat Oncol Biol Phys* 1997, **39**, 831–836.
9. Withers HR, Peters LJ, Taylor JM, *et al.* Local control of carcinoma of the tonsil by radiation therapy: an analysis of patterns of fractionation in nine institutions. *Int J Radiat Oncol Biol Phys* 1995, **33**, 549–562.
10. Bourhis J, Wibault P, Lusinchi A, Bobin S, Luboinski B, Eschwege F. Status of accelerated fractionation radiotherapy in head and neck squamous cell carcinomas. *Curr Opin Oncol* 1997, **9**, 262–266.
11. Maciejewski B, Majewski S. Dose fractionation and tumour repopulation in radiotherapy for bladder cancer. *Radiother Oncol* 1991, **21**, 163–170.
12. Roberts SA, Hendry JH. The delay before onset of accelerated tumour cell repopulation during radiotherapy: a direct maximum-likelihood analysis of a collection of worldwide tumour-control data. *Radiother Oncol* 1993, **29**, 69–74.
13. Trott KR, Kummermehr J. Rapid repopulation in radiotherapy: a debate on mechanism. Accelerated repopulation in tumours and normal tissues. *Radiother Oncol* 1991, **22**, 159–160.
14. Fowler JF. The phantom of tumor treatment—continually rapid proliferation unmasked. *Radiother Oncol* 1991, **22**, 156–158.
15. Kummermehr J, Dorr W, Trott KR. Kinetics of accelerated repopulation in normal and malignant squamous epithelia during fractionated radiotherapy. *Br J Radiol* 1992, **24**, 193–199.
16. Dubben HH. No clinical evidence for the influence of overall treatment time on TCD50 of head and neck tumours. *Radiother Oncol* 1992, **25**, 142–143.
17. Lai PP, Pilepich MV, Krall JM, *et al.* The effect of overall treatment time on the outcome of definitive radiotherapy for localized prostate carcinoma: the Radiation Therapy Oncology Group 75-06 and 77-06 experience. *Int J Radiat Oncol Biol Phys* 1991, **21**, 925–933.
18. Begg AC. The clinical status of T_{pot} as a predictor? Or, why no tempest in the T_{pot}! *Int J Radiat Oncol Biol Phys* 1995, **32**, 1539–1541.
19. Hoyer M, Jorgensen K, Bundgaard T, *et al.* Lack of predictive value of potential doubling time and iododeoxyuridine labelling index in radiotherapy of squamous cell carcinoma of the head and neck. *Radiother Oncol* 1998, **46**, 147–155.
20. Tsang RW, Fyles AW, Kirkbride MB, *et al.* Proliferation measurements with flow cytometry T_{pot} in cancer of the uterine cervix: correlation between two laboratories and preliminary clinical results. *Int J Radiat Oncol Biol Phys* 1995, **32**, 1319–1329.
21. Sham E, Durand RE. Cell kinetics and repopulation mechanisms during multifraction irradiation of spheroids. *Radiother Oncol* 1998, **46**, 201–207.
22. Sham E, Durand RE. Repopulation characteristics and cell kinetic parameters resulting from multifraction irradiation of xenograft tumors in SCID mice. *Int J Radiat Oncol Biol Phys* 1999, **43**, 617–622.
23. Friedl F, Kimura I, Osato T, Ito Y. Studies on a new human cell line (SiHa) derived from carcinoma of uterus. I. Its establishment and morphology. *Proc Soc Exptl Biol Med* 1970, **135**, 543–545.
24. Begg AC, McNally NJ, Shrieve DC, Kärcher H. A method to measure the duration of DNA synthesis and the potential doubling time from a single sample. *Cytometry* 1985, **6**, 620–626.
25. White RA, Terry NHA, Meistrich ML, Calkins DP. Improved method for computing potential doubling time from flow cytometric data. *Cytometry* 1990, **11**, 314–317.
26. Durand RE. Determining T_{pot} in heterogeneous systems: a new approach illustrated with multicell spheroids. *Cytometry* 1993, **14**, 527–534.
27. Steel GG. *Growth Kinetics of Tumours*. Oxford, Oxford University Press, 1977.
28. Durand RE. Tumor repopulation during radiotherapy: quantitation in two xenografted human tumors. *Int J Radiat Oncol Biol Phys* 1997, **39**, 803–808.
29. Eschwege F, Bourhis J, Girinski T, *et al.* Predictive assays of radiation response in patients with head and neck squamous cell carcinoma: a review of the Institute Gustave Roussy experience. *Int J Radiat Oncol Biol Phys* 1997, **39**, 849–853.
30. Begg AC, Hofland I, Kummermehr J. Tumour cell repopulation during fractionated radiotherapy: correlation between flow cytometric and radiobiological data in three murine tumours. *Eur J Cancer* 1991, **27**, 537–543.
31. Hansen O, Grau C, Bentzen SM, Overgaard J. Repopulation in the SCCVII squamous cell carcinoma assessed by an *in vivo—in vitro* excision assay. *Radiother Oncol* 1996, **39**, 137–144.

32. LaRue SM, Fox MH, Withrow SJ, *et al*. Impact of heterogeneity in the predictive value of kinetic parameters in canine osteosarcoma. *Cancer Res* 1994, **54**, 3916–3921.
33. Speke AK, Hill RP. Repopulation kinetics during fractionated irradiation and the relationship to the potential doubling time, Tpot. *Int J Radiat Oncol Biol Phys* 1995, **31**, 847–856.

Acknowledgements—The skillful technical assistance of Nancy LePard, Denise McDougal and Darrell Trendall is gratefully acknowledged. This study was supported by USPHS grant CA-56600 from the National Cancer Institute and by the Medical Research Council of Canada.