Eur J Cancer, Vol. 27, No. 5, pp. 537–543, 1991.
Printed in Great Britain

0277–5379/91 $3.00 + 0.00
Pergamon Press plc

Papers

Tumour Cell Repopulation during Fractionated Radiotherapy: Correlation between Flow Cytometric and Radiobiological Data in Three Murine Tumours

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This study tested whether the potential doubling time of tumour cells measured before or during treatment could predict the repopulation rate of surviving clonogens during fractionated radiotherapy. Tumours used for the study were a fibrosarcoma (SSK 2), an adenocarcinoma (AT 7) and a squamous cell carcinoma (AT 478), all grown subcutaneously in the C3H mouse. Potential doubling times (T_{pot}) were measured using the thymidine nanalogue iododeoxyuridine (IUdR) and flow cytometry. Results were compared with previous radiobiological studies on these tumours in which repopulation rates during radiotherapy were estimated using the tumour growth delay and tumour cure assays. Fractionated treatments consisted of daily doses of 4 or 8 Gy to clamped (hypoxic) tumours, 6 days per week for 1–3 weeks. T_{pot} values increased markedly during therapy for two of the tumours (SSK 2 and AT 478), by a factor of more than 10 for AT 478 in the third treatment week. T_{pot} remained approximately constant for the third tumour (AT 7). In no case was there evidence from the labelling studies of a shortening of T_{pot} which would suggest accelerated repopulation. From the radiobiological data, effective clonogen doubling times during radiotherapy were calculated from the doses required to produce a given effect in short and long treatment schedules. In the second week of treatment, effective clonogen doubling times in two tumours were approximately equal to the pretreatment T_{pot}, and shorter than the pretreatment T_{pot} in the third tumour. At some time during treatment, the surviving clonogens in these tumours therefore proliferated at the same rate or faster than before treatment. The difference between the labelling and radiobiological measurements was ascribed to the fact that, shortly after the start of a fractionated treatment, the IUdR labelling technique measures primarily doomed cells. These results show that kinetic measurements using DNA labelling techniques made during fractionated radiotherapy in most cases do not reflect the proliferation status of the surviving cells which are responsible for treatment outcome. Pretreatment T_{pot} measurements give a much better indication of the proliferation rate of surviving cells but in some cases may underestimate repopulation during radiotherapy.
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INTRODUCTION

ONE OF THE factors affecting the probability of cure in fractionated radiotherapy is repopulation of surviving tumour cells between fractions [1–3]. In head and neck tumours in particular, evidence is accumulating that repopulation is a frequent cause of treatment failure for long schedules of 6–7 weeks. This evidence includes the poor results with split course therapy and the significantly higher doses required to achieve a particular tumour control probability as the overall treatment time is prolonged [1–5] and the early promise of very short fractionation schemes such as the 12 day "CHART" schedule [6]. The problem of repopulation during fractionated radiotherapy will be less acute in tumours with lower proliferation potential. Such tumours will occur not only at specific sites (e.g. adenocarcinoma of the breast) but also within each histological type, including head and neck. Variability in repopulation rates means that only a proportion of tumours within a class will be at risk from conventional (prolonged) fractionation schemes. An assay which can predict which tumours are at risk would aid the radiotherapist in choosing an optimum schedule.

One assay which can measure the proliferation potential of tumours in the patient involves a single injection of the thymidine analogue iododeoxyuridine (IUdR) or bromodeoxyuridine (BUdR), followed several hours later by a tumour biopsy in which analogue incorporation into DNA is measured by a monoclonal antibody and flow cytometry [7–9]. In this way the tumour's potential doubling time (T_{pot}, a parameter which combines the cell cycle time and the growth fraction [10] can be estimated [11]. Mean T_{pot} values measured with this technique in untreated head and neck tumours [7, 9, 12, 13] are in

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Revised 23 Jan. 1991; accepted 8 Feb. 1991.

reasonably good agreement with estimated mean doubling times of cells during fractionated radiotherapy, estimated from clinical data of cure dose (TCD50) for different overall treatment times [3]. This indirect evidence suggests that the pretreatment T_{pot} could be used to predict repopulation rates during fractionated radiotherapy, at least for head and neck tumours, as suggested by Trott and Kummermehr [2].

The primary purpose of the present studies was to establish if this relationship could be confirmed in three mouse tumour models with different growth rates. The second question addressed was whether kinetic measurements could be made during radiotherapy, rather than pretreatment, to get a better estimate of proliferation of surviving cells during radiotherapy. Lastly, the relationship between the change in labelling index (LI; proportion of cells labelled with the analogue) and radiosensitivity was also investigated.

For these studies, a collaboration was set up between the radiobiology groups of Amsterdam (The Netherlands Cancer Institute), with experience of the kinetic methods, and Munich (GSF), with experience of the repopulation patterns of several rodent tumours during radiotherapy. Three tumours with different histologies, doubling times and radiosensitivities were selected for the studies. A more detailed report of the functional proliferation studies will be published (J.K. *et al.*).

MATERIALS AND METHODS

Mice and tumours

The origin and characteristics of the three transplantable mouse tumours chosen for the present study are summarised in Table 1. The choice of a fibrosarcoma, a mammary tumour and a squamous cell carcinoma ensured representation of diverse classes of solid tumour. All tumours were grown subcutaneously in the flank by transplantation of approximately 1 mm^3 tumour fragments according to previously described procedures [14]. None of the three tumours has shown evidence of immunogenicity (unpublished results). Tumours were used in passages 9, 10, and 25 for AT 7, AT 478 and SSK 2, respectively. The tumours were entered for treatment after reaching a diameter of approximately 7 mm.

Irradiations

All irradiations were carried out using a Seifert Isovolt 320 X-ray set operating at 300 kVp with a 0.6 mm Cu + 1.0 mm Al filter giving a dose rate of 5.5 Gy per minute. The mice were anaesthetised for irradiation using hexabarbital-Na (120 mg/kg). The tumours were clamped 5 min before and during each irradiation using a plastic clamp with a preset 0.5 mm gap to occlude the blood supply and render all cells temporarily hypoxic [14]. This ensured uniform radiosensitivity of the irradiated cells, avoiding problems of reoxygenation and allowing comparison with previous experiments [14].

Table 1. *Tumour characteristics*

	SSK2	AT 7	AT 478
Histology	Fibrosarcoma	Adenocarcinoma	Squamous cell carcinoma
Origin	MCA*	Spontaneous	Spontaneous
Host	C3H	C3H	C3H
Differentiation	Poor	Poor	Moderate

*Methylcholanthrene-induced; cloned.

Iododeoxyuridine (IUdR)

IUdR powder (Sigma) was dissolved in 0.9% saline to which 0.1 mol/l NaOH was added to bring the pH up to approximately 10.5, since IUdR is less soluble at neutral pH. After dissolving, the pH was brought to pH 8.5 or lower for injection into the mice. No irritation or acute reaction was observed at this pH. The concentration was adjusted to 1.5 mg/ml and 0.02 ml per g mouse was injected intraperitoneally, giving an injected dose of 30 mg/kg.

Staining and flow cytometry

After excision, the tumours were halved, fixed in 70% ethanol and stored in the dark at 4°C until staining. The preparation of the tissue and staining with the anti-IUdR monoclonal antibody for flow cytometry has been described previously [7]. Briefly, the fixed tumour pieces were cut into small pieces and incubated in pepsin to produce a suspension of nuclei [15]. This was followed by an acid denaturation step, incubation with the mouse monoclonal antibody, incubation with an FITC-conjugated anti-mouse antibody and addition of propidium iodide (PI) to stain total DNA. This pepsin method produced yields of 2–3 $\times 10^8$ nuclei per gram for untreated tumours. For irradiated tumours, the nuclear yields decreased to a minimum of 0.7–1.0$\times 10^8$, reduction by a factor of between 2 and 4.

Flow cytometry was carried out using a FACScan or FACS IV flow cytometer (Becton Dickinson, Belgium) using excitation at 488 nm and collecting the green (FITC; IUdR) and red (PI; DNA) fluorescence signals using the following filters: (a) FACS IV: 520–540 nm band pass (green), 620 nm long pass (red); (b) FACScan: 515–545 nm band pass (green), 650 nm long pass (red).

For the calculation of the T_{pot}, both the DNA synthesis time, T_s, and the labelling index, LI, are necessary ($T_{pot} = \lambda T_s/LI$). The age distribution factor λ was chosen to be unity for consistency with our clinical studies where this assumption is made [13]. It is possible that a value around 0.8 would be more realistic. This would mean that we were overestimating T_{pot} by around 25%, but the relative changes observed during fractionation will remain unaltered. T_s was calculated by first estimating the parameter relative movement (RM) using a window placed around the undivided labelled cells and measuring their position relative to G1 cells [11]. T_s was then calculated according to the equation

$$T_s = 0.5 \times t/(RM - 0.5)$$

where t is the time between IUdR injection and tumour excision.

Experimental protocol

The outline of the experimental plan is shown in Table 2. Each tumour type was given a fractionated irradiation schedule consisting of daily fractions six times per week, lasting for 2 or 3 weeks. All tumours were clamped at the time of irradiation. The dose per fraction was 8Gy for AT 478 and SSK 2, and 4 Gy for AT 7, since the latter tumour was considerably more radiosensitive than the other two. IUdR was given to groups of mice at various times throughout the fractionation schedule. Each group consisted of 4 or 5 mice. The IUdR was always given in place of the next X-ray fraction, e.g. for the two fraction point, IUdR was given instead of the third fraction of day 3, 24 h after the last fraction; for the six fraction point, IUdR was given instead of the seventh fraction on day 8, 48 h after the last fraction (see Table 2).

Table 2. Experimental protocol

SSK2	
S	Control
XXXXS	4F
XXXXXXoS	6F
XXXXXXoXXXS	9F
AT7	
S	Control
XXS	2F
XXXXS	4F
XXXXXXoS	6F
XXXXXXoXXXS	9F
AT 478	
S	Control
XXXS	3F
XXXXXXoS	6F
XXXXXXoXXXS	9F
XXXXXXoXXXXXXoS	12F
XXXXXXoXXXXXXoXXXS	15F

X = X-irradiation; o = no irradiation (treatment gap); S = sample: tumour excision 4 h after IUdR injection. All intervals were 24 h (X-X; X-o; o-X; o-S; X-S). Dose/fraction: 4 Gy (AT 7), 8 Gy (SSK 2, AT 478). Tumours clamped (hypoxic) during irradiations.

For each tumour type, a group of untreated mice was also given IUdR to allow comparison of kinetics before and during irradiation. For all groups, irradiated or unirradiated, mice were sacrificed 4 h after the injection of IUdR, a time regarded as optimal for the measurement of both T_s and LI. The LI was corrected for the divided labelled cells, i.e. those reappearing at the G1 position, by halving the number of these cells for the LI calculation [16].

The protocol described above followed that of previous experiments designed to study repopulation during therapy by tumour response, using growth delay as endpoint [17–19]. In these experiments, 6 fractions were given either in 1.5 days (minimum of 4 h between fractions) or daily for 6 days. A test dose was then given the same day as the last fraction for the short scheme (day 1.5), or on day 7 for the long scheme, resulting in a 5.5 day time difference between schedules. To investigate repopulation in the second week of fractionation, all tumours received 6 daily fractions in the first week followed by either the short or the long fraction scheme in the second week. A similar design was used to investigate repopulation in the third week, i.e. two weeks of daily fractionation followed by either a short or long scheme in the third week. A functional measure of repopulation was obtained from the difference in isoeffective test doses between the long and short schemes, a higher test dose being required in the longer schedule to offset repopulation. The radiobiological studies were carried out over a number of years, before the kinetic studies were initiated.

Growth delay analysis

The effective clonogen doubling times were calculated from growth delay dose response data, i.e. effectively from the dose differences between short and long schedules. These data were analysed using a direct analysis method which uses data at all dose levels and not simply at a single isoeffect. This is similar to the approach of Thames *et al.* [20]. The principle for growth delay data has been described in more detail in the recent publication of Guttenberger *et al.* [14]. Briefly, the assumptions are that, firstly, all levels of effect are described by a general LQ (linear-quadratic) model, and secondly, effects can be translated into growth delays by a link function, in this case a power function. Non-linear regression was then applied, leading to mean values and errors on parameter estimates, including that of number of fractions lost through repopulation. This could then be converted into a doubling time knowing the radiosensitivity parameters (α and β) and the time period over which these fractions were lost, i.e. the 5.5 days between the long and short schedules.

RESULTS

DNA histograms for all the three types of untreated tumour showed two major peaks consistent with a peak of normal, non-malignant cells and a peak of higher DNA content representing the tumour cells (Fig. 1). The ploidy of the tumour cells was calculated to be 2.1, 2.0 and 1.9 for SSK 2, AT 7 and AT 478 tumours respectively.

The effect of fractionated irradiation on the DNA distributions is shown for AT 478 tumours in Fig. 2. With increasing number of 8 Gy (hypoxic) fractions administered, an increase in the tumour G2M fraction is evident (relatively larger right hand peak), reaching a maximum after 6 to 9 fractions. This is consistent with the known G2 blocking action of radiation. The histograms for four tumours in each group are shown, which demonstrate the magnitude of intertumour variations. Some variation was seen between tumours in a particular group, although this intertumoral variation was too small to obscure differences between groups.

Figure 3 shows examples of the red vs. green fluorescence labelling patterns for AT 7 tumours excised 4 h after IUdR labelling, for both control and irradiated tumours. The labelled cells could be clearly distinguished from the unlabelled cells. Two populations of labelled cells were seen, one at the tumour G1 position and the other nearer the tumour G2 position,

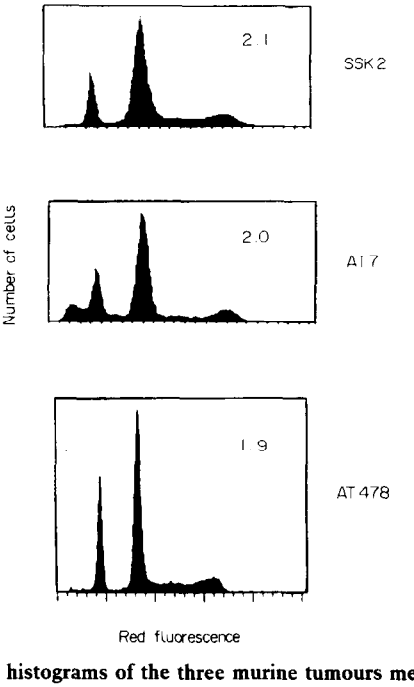

Fig. 1. DNA histograms of the three murine tumours measured by flow cytometry using red fluorescence after propidium iodide staining. The fluorescence ratios of the first and second peaks (normal G1 and tumour G1 cells respectively) are given, representing the DNA index of the tumour cells (2.0 = tetraploid).

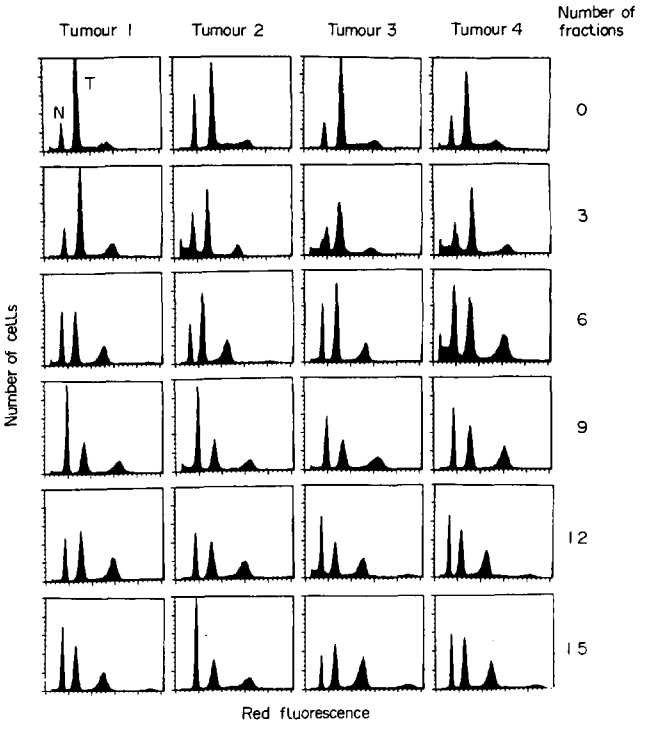

Fig. 2. Change in the cycle phase distributions of AT 478 tumours during fractionated radiotherapy. Each row shows DNA histograms of four tumours within the same treatment group. Histograms were measured after treatment with different numbers of 8 Gy (clamped) fractions, ranging from 0 (top row; controls) to 15 (bottom row). Peaks represent, from left to right, normal cells, tumour G1 and tumour G2 cells.

consistent with a fraction of the cells originally labelled in late S having traversed G2 and mitosis to appear in G1. The data in Fig. 3 (right panel) were taken from tumours given 4 fractions of 4 Gy, at which time little evidence of a G2 block was seen in the DNA histograms. For tumours showing a clear G2 block i.e. after higher doses, relatively fewer labelled cells appeared above G1. For example, in untreated AT 478 tumours 6 h after IUdR injection, approximately 41% of the labelled cells were above G1, whereas only 17% of labelled cells reached G1 for tumours labelled after 6 fractions of irradiation, when a significant G2 block could be seen (data not shown).

Determining T_s involved measurement of the position (mean red fluorescence) of the undivided labelled population [11]. Labelled cells were also seen in the irradiated tumours (right panel), although a window analysis showed that these were fewer than in controls (see below). Significant movement through the cell cycle in the 4 h between injection and excision was also seen in the irradiated tumours. Differences between tumours in one group were again small, indicating the reproducibility of the model and the labelling technique.

The cell kinetic parameters derived from these flow cytometry data for irradiated and unirradiated tumours are shown in Fig. 4. For all three tumour types the LI decreased markedly during the first few fractions, followed either by an incomplete recovery (AT 7) or a further decrease at a much reduced rate (SSK 2, AT 478) (Fig. 4a). After 3 or 4 fractions, the LI decreased by 76%, 58% and 65% relative to that immediately before irradiation, for SSK 2, AT 7 and AT 478 tumours respectively. The LI remained lower than controls for all tumours throughout the irradiation schedule.

The DNA synthesis time, T_s, did not show large changes as a result of irradiation (Fig. 4b). There was a tendency for T_s to increase slightly for AT 478 and to decrease for AT 7, while values for SSK 2 remained approximately constant. The changes in the potential doubling time, a combination of the LI and T_s, are shown in Fig. 4c. Two of the tumours, SSK 2 and AT 478, showed a marked and progressive increase in T_{pot} during fractionation. For AT 7, the LI decrease was offset by the decrease in T_s, resulting in little change in the calculated T_{pot}. Taken at face value, therefore, these data imply a decrease in repopulation rate during fractionation, at least for two of the three tumour types.

These data can be compared with repopulation in the tumours during therapy measured radiobiologically. The irradiation protocols consisted of a fixed number of radiotherapy fractions given in either a short or long overall time, preceded or not by one or two weeks of daily X-ray fractions (see Materials and Methods). This was followed by graded single test doses to bring the response into a suitable range for the growth delay assay. For all three weeks of fractionation, more dose was required to produce a given effect with the longer schedules. The displacement between the curves was taken as a measure of the repopulation occurring in 5.5 days, the time difference between the short and long schedules.

From the simultaneous analysis including all dose points, the parameter α/β and the number of dose fractions repopulated in

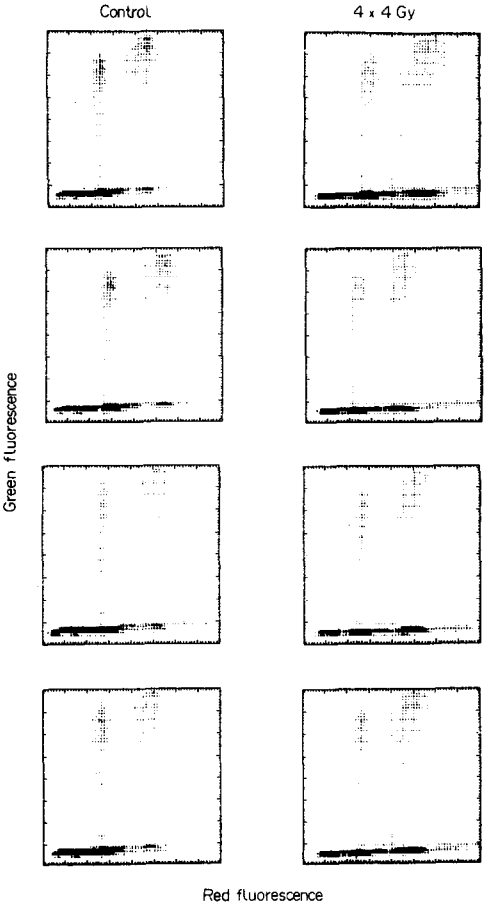

Fig. 3. Cytograms of IUdR content (green fluorescence) vs. total DNA content (red fluorescence) for AT 7 cells from tumours excised 4 h after IUdR injection. Left and right panels show results for untreated tumours and for tumours labelled after treatment with 4 fractions of 4 Gy (clamped), respectively.

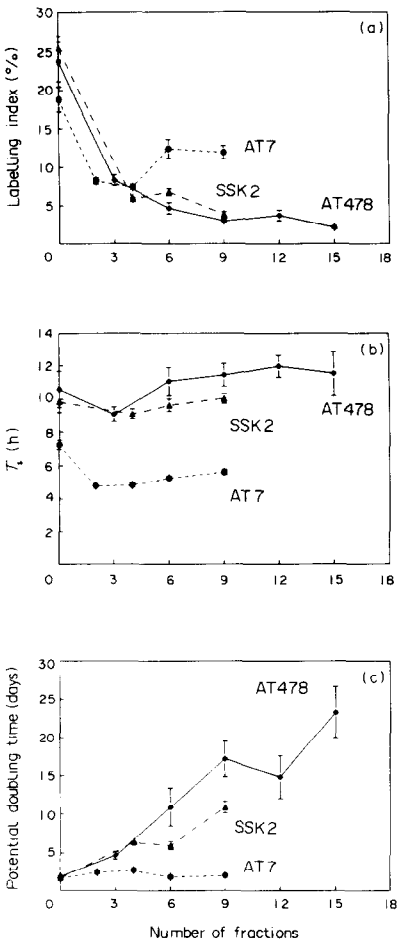

Fig. 4. Change in kinetic parameters during fractionated radiotherapy for three murine tumours, measured by IUdR labelling and flow cytometry. (a), (b) and (c) panels show data on LI, T_s and T_{pot} respectively. The tumour type is indicated against each curve. 4–5 tumours per point.

each week were estimated. Absolute values of α and β were also estimated from the single dose TCD50 (Table 3) after first calculating the number of clonogenic cells per 100 mg tumour. This was done by dividing the estimated total cell number per tumour by the TD50 (number of cells required to produce 50% of tumours in inoculated sites). These calculations allowed

Table 3. Tumour take (TD50), tumour cure (TCD50) and radiosensitivity data

Tumour	TD50 (cells)	TCD50 (Gy)	Clonogens at risk*	α (Gy^{-1})†	β (Gy^{-2})†	α/β (Gy)
SSK 2	1×10^1	73	10^7	0.074	0.0021	35.3+4.6
AT 7	2×10^2	45	5×10^4	0.142	0.0024	59.5±16.9
AT 478	5×10^2	68	2×10^5	0.059	0.0019	31.5±8.7

TD50 = take dose 50%: number of cells required to produce tumours in 50% of inoculated sites (data of J.K. 1985, unpublished).
TCD50 = Tumour control dose 50%: single X-ray dose required to control 50% of tumours treated under hypoxic conditions.
*Tumour cells per tumour/TD50.
†Linear and quadratic coefficients in dose-effect (E) relationship: $E = \alpha.d+\beta.d^2$ where d is radiation dose (hypoxic). Data obtained from isodase cure data for different fractionation schedules using equal doses per fraction.

Table 4. Repopulation parameters

Tumour	Pretreatment T_{pot}(d)	Pretreatment T_D(d)	Week of fraction-ation	Clonogen doublings†	T_{rep}† (d)
SSK 2	1.5	1.5	1	4.9(4.6–5.2)	1.1(1.0–1.2)
			2	3.6(3.1–4.0)	1.6(1.4–1.8)
AT 7	1.8	2.0	1	0.8(0.4–1.3)	6.5(4.2–14.5)
			2	2.4(1.7–3.0)	2.3(1.6–3.2)
AT 478	1.9	3.5	1	1.4(1.0–1.8)	3.9(3.0–5.5)
			2	4.1(3.6–4.6)	1.3(1.2–1.5)
			3	5.4(4.4–6.3)	1.0(0.9–1.2)

*Calculated from the differences in dose response data for short and long schedules, and the radiosensitivity parameters (see Materials and Methods).
†Effective doubling time of surviving clonogens during treatment calculated from the number of clonogen doublings and the time difference in schedules (5.5 days).

estimates of surviving fraction to be made at the TCD50 dose (assuming average cell number surviving at TCD50 is 0.693), from which, knowing the α/β ratio, absolute values of α and β could be derived. The number of repopulated fractions could then be converted into number of clonogen doublings during the extra 5.5 day interval. From this the effective clonogen doubling time could simply be calculated from

$$-5.5 = \ln 2/(\ln SF \times T_{rep})$$

where SF is the survival rate per dose fraction and T_{rep} is the number of repopulated fractions.

The results of these calculations are shown in Table 4, together with the pretreatment measurement of T_{pot} from the IUdR technique and the pretreatment volume doubling time. By the second week, all three tumours had effective doubling times of surviving cells less than or close to the pretreatment volume doubling time. Apart from week 1 for AT 7, there was little evidence of a decrease in repopulation rate during radiotherapy. The most complete data were obtained for AT 478 where it was clear that the effective clonogen doubling times became shorter than both the volume doubling time and the pretreatment T_{pot} during the second and third weeks of daily fractionation.

Finally, the relationship between reduction in LI and radiosensitivity was sought. AT 478 and SSK 2 had approximately the same radiosensitivity, as judged by both TCD50 and specific growth delay, and the decrease in LI was also similar (Fig. 4a). AT 7 was significantly more radiosensitive both in terms of tumour cure and specific growth delay (growth delay/doubling time). A lower dose per fraction was therefore used in the present experiments. After this lower dose, the LI decrease was less than for the other tumours (Fig. 4a). With a higher dose, the LI decrease would have been greater, although whether it would exceed that for the other tumours at the same dose is not known.

DISCUSSION

The purpose of this study was to investigate the validity of measuring the potential doubling time, T_{pot}, before or during treatment, as a measure of the repopulation rate of clonogenic tumour cells during fractionated radiotherapy. Values for T_{pot}

were measured using a single sample after injection of IUdR, as is being used in the clinic [7, 9, 12, 13]. Two main conclusions can be drawn from the results. Firstly, the correlation between the flow cytometry measurements made during treatment and the radiobiological measurements was poor. Secondly, the surviving tumour clonogens in some instances appeared to be capable of proliferating during radiotherapy faster than indicated by the pretreatment T_{pot}.

Comparison of methods to measure repopulation

The lack of correlation between the repopulation rates measured by the two techniques is evident by comparing the T_{pot} values (Fig. 4c) with the radiobiologically measured clonogen doubling times (Table 3). By the middle of the second week, SSK 2 and AT 478 tumours had reduced LIs giving mean T_{pot}s which were 6–10 times higher (slower repopulation) than untreated tumours. The radiobiological studies, however, showed either little change in doubling time (SSK 2) or shorter doubling times (AT 478) than either the pretreatment volume or potential doubling times, indicating accelerated rather than decelerated repopulation. The discrepancy is likely to be due to the fact that the radiobiological methods of growth delay and tumour control measure only the clonogenic cells, while the IUdR technique measures all intact cells in the tumour without discriminating between surviving and doomed cells. The fractionation studies *in vivo* which have been carried out with the three tumour models allow reasonable estimates of cell killing to be made for a given dose (see Results). Estimates of survival for the hypoxic irradiations of 4 Gy (AT 7) or 8 GY (AT 478 and SSK2) were all around 0.55, being approximately equivalent to doses of 1.5 and 3 Gy respectively under oxic conditions. With this level of cell killing, approximately 90% of the cells will be dead or doomed after 3 fractions, i.e. the middle of the first week. After 9 fractions, the middle of the second week, only 1 in approximately 500 cells will be clonogenic. The IUdR technique will therefore primarily measure labelling in doomed cells. It is apparent that these measurements do not represent the behaviour of the relatively few survivors that determine the outcome of treatment.

The repopulation rate of the survivors exceeded, at times, that given by the pretreatment T_{pot}. The rate of doubling is determined by the cell cycle time (T_c), the fraction of cells in the proliferating compartment (GF) and the rate of cell loss (Φ; [10]). T_{pot} represents a combination of T_c and GF, i.e. the cell number doubling time in the absence of cell loss. A clonogen doubling time less than T_{pot} implies not only the elimination of cell loss for this cell population, but also a concomitant increase in the growth fraction compared with untreated tumours. The results reported here for AT 478 tumours therefore imply a doubling time approaching that of the cell cycle time in the second and third weeks of treatment. The relative hypervascularity often observed in regressing tumours may be one explanation of this phenomenon [21]. Tumours in the present study were clamped during irradiation to enable comparison with previous studies in which radiosensitivity changes due to hypoxic fraction changes could be avoided by using this method. It should be remarked that the location of survivors with respect to blood vessels, and thus sites for repopulation, will be different than for ambient irradiations when survivors will occur primarily in the naturally hypoxic regions. It is also likely, however, that with the schedules employed here, extensive reoxygenation will take place, leaving the majority of cells oxic. The comparison made here of labelling and radiobiology techniques remains valid in any case, since irradiation conditions were held the same for both.

If human tumours undergoing radiotherapy react in a similar way to these mouse tumours, it is possible that pretreatment T_{pot} measurements may actually underestimate clonogen repopulation rates in some instances. The analysis of Withers and colleagues [3] has shown that, at least in the latter part of fractionated radiotherapy for head and neck tumours, the average clonogen doubling time is of the order of a few days, i.e. close to the average pretreatment T_{pot} value [7, 13] and considerably shorter than the average tumour volume doubling time (T_D) of 2 months or longer. From these clinical data and the present animal tumour data, it is clear that the pretreatment volume doubling time is a poor predictor of repopulation during treatment. T_{pot} and T_c would be better predictors of intratreatment repopulation, but which of these is better for human tumours is not yet resolved. It is not possible with present kinetic techniques to measure the cell cycle time with one or two samples. This can only be done with multiple samples, a situation impossible to realise in routine clinical practice. An independent measure of growth fraction, e.g. with an antibody such as Ki67 [22], would allow T_c to be derived from the T_{pot}. No reliable antibody technique to measure growth fractions in solid human tumours is currently available.

LI changes

Ramsay and colleagues [23] investigated changes in LI in a mouse fibrosarcoma during fractionated irradiation using BUdR. They showed that the decrease in tumour LI after 5 fractions predicted for cure when comparing daily or twice per day fractionation schedules. They therefore concluded that LI changes after irradiation could indeed be used as a measure of repopulation occurring during radiotherapy. They also stated, however, that absolute repopulation rates could not be estimated from LI values during radiotherapy because of the problem of doomed cells; only relative estimates of repopulation could be made for the different fractionation schedules. In our experiments, there was not a one to one correlation between the LI during radiotherapy and clonogen repopulation rate, since SSK 2 and AT 478 showed similar patterns and degree of decrease in LI whereas, of these two, AT 478 showed the more marked changes in clonogen doubling time. It is concluded that LI changes could be used only as a rough guide, at best, to repopulation during radiotherapy, and then in an indirect manner since the LI decreases were associated with repopulation rate increases. Such LI changes (assessed by *in vitro* labelling) have been used by some groups to predict outcome (e.g. [24]), an application of LI measurements essentially unrelated to the monitoring of proliferation.

Assay problems

The conclusions drawn from these data depend on the assumption that the only factor responsible for the growth delay differences between the long and short schedules is repopulation. Incomplete repair could be a possible confounding influence which would disproportionately increase the effect of the short schemes. This is unlikely to be a large factor, however, since an interval of at least 4 h was left between treatments, after which repair is effectively complete at these doses [14]. Another factor which could affect response is induced synchrony. Blocks in G2 were demonstrated (Fig. 2), although they will occur for both short and long schemes. In addition, it is not known whether the tiny minority of surviving cells will undergo a G2 block

similar to the doomed cells. The precise influence of synchrony is therefore impossible to assess from these data.

The conclusions concerning comparisons with the flow cytometric measurement of T_{pot} assume an accurate measurement of this parameter both before and after irradiation. The one sample IUdR method used here may give erroneously short T_s estimates if the sampling time is less than a G2 period [16]. At lower doses this was not the case, since some labelled cells appeared in G1 during the 4 h interval used. At higher doses a pronouced G2 block was seen and this could have disturbed (shortened) the T_s estimates. The method of calculating T_s from the "relative movement" (RM) of labelled cells also depends on an even distribution of cells throughout the S phase, i.e. that RM at time zero is 0.5, half way between G1 and G2. Deviations from an even S distribution should be observable in a skewness of the DNA histogram between G1 and G2. In some histograms a slight upward slope was evident accompanying the G2 block after several fractions of radiation, implying an initial RM of greater than 0.5. The estimated T_s value may therefore be too short for some irradiated samples. This does not appear to have been a large or consistent problem, however, since the T_s values did not change dramatically during treatment (Fig. 4b). This suggests that the cells progressed relatively normally through S despite a block in G2. A further conclusion is that, because of the G2 block and the effects mentioned above, T_{pot} will probably be underestimated by only observing the labelled cells (proportion and rate of progression through S), further increasing the discrepancy between the kinetic and radiobiological measurements.

Conclusions

The present studies have shown that T_{pot} values can be measured both before and during radiotherapy using a single sample taken after IUdR injection, but that the changes in this kinetic parameter during treatment do not correlate with kinetic changes undergone by the surviving clonogens. Of the three kinetic parameters that are candidates for correlation with the rate of clonogen repopulation during fractionated radiotherapy, namely T_c, T_{pot} and T_D, T_{pot} has the advantages that (a) it can be easily measured in human tumours and (b) it appears to provide a better measure of repopulation during radiotherapy than T_D. It could, however, underestimate clonogen repopulation in some tumours. T_c cannot at present be measured in human tumours with one or a few samples.

The pretreatment T_{pot} may therefore not be an ideal predictor of clonogenic repopulation during radiotherapy but, overall, it is the best parameter available at present. Is predictive value in human tumours will only be answered by application within randomized clinical trials, such as the present European trial of accelerated versus conventional fractionation in advanced head and neck tumours (EORTC trial 22851) in which a proportion of the tumours have been labelled with IUdR [13]. Information is still needed on the time at which clonogens begin their rapid repopulation during radiotherapy in tumours of different types, and what factors determine both the time and the rate. Such knowledge would help the more rational design of radiotherapy schedules for individual tumours.

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Acknowledgements—This work was partly supported by a grant from the Deutsche Forschungsgemeinschaft (KU 576/2-2).