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INFLUENCE OF TREATMENT TIME ON THE RESPONSE OF RAT RHABDOMYOSARCOMA R1H TO FRACTIONATED IRRADIATION

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Abstract

The aim of this work was to study influence of overall treatment time on response of an experimental tumour to fractionated radiotherapy. Rhabdomyosarcoma R1H of the rat was treated by fractionated irradiation applying 30 fractions in different overall treatment times of 10, 18.5, 29, 39, and 67 days. The doses per fraction ranged from 1.50 to 2.67 Gy. Tumour response was assessed by net growth delay and by an in vitro colony assay. The results indicate that repopulation of the tumour with clonogenic tumour cells slowed down during treatment leading to an average doubling time of clonogenic tumour cells of 8 ± 1 days, which is considerably longer than that of unirradiated tumours (3 days). The repopulation rate seemed to increase after about 3 weeks of treatment with a doubling time of tumour clonogens of 5 ± 1 days, which was still lower than in control tumours.

Key words: Irradiation, fractionation, rhabdomyosarcoma, rat, growth delay, colony assay, repopulation, overall treatment time.

One of the main factors that determines success of fractionated radiotherapy is the repopulation of surviving tumour cells during treatment (1, 2). Data from animal experiments (3–8) and clinical data (9–16) suggest that the increase in the number of clonogenic tumour cells between the fractions may result in failure to control the tumour in some fractionated treatment regimens (17–19). As a consequence, accelerated fractionation, i.e. reduction of the overall treatment time while keeping the dose per fraction constant, has been discussed as a new possibility to improve radiotherapy for some tumour types (20–23).

In normal tissues, accelerated repopulation after radiation-induced depletion of cells is a well known homoeostatic phenomenon (24). Proliferation does not occur in normal tissues up to a certain time after start of irradiation. For longer times, the total dose required to achieve a given isoeffect rises steeply when compensatory proliferation begins. This was shown by Denekamp (25), who observed a need for increasing the total dose after 2 and 3 weeks of multifraction treatment. This corresponds approximately to the time when the desquamative skin reaction is observed after a single dose of x-rays. Both effects reflect the normal turnover time of the basal layer cells in the epithelium.

In tumours, the role of proliferation in the response to irradiation is still not very well understood, mainly because several mechanisms are superimposed. This is particularly relevant for fractionated irradiation.

After single doses of irradiation, accelerated repopulation has been shown for various experimental tumour systems (5, 16, 22, 26–28) and it appears that some general patterns of proliferation govern the response to cytotoxic treatment. Carcinomas show a quicker response to treatment than sarcomas, which was found to be due to the higher cell loss rates in carcinoma (5, 24). Furthermore, the degree of cell kill seems to play an important role as far as the time of onset and the rate of repopulation are concernced (7, 26). After single dose treatments, many tumours show a biphasic pattern of repopulation characterized by a reduced initial growth rate or even cessation of growth which is subsequently followed by accelerated repopulation (26, 28–30). In general, the duration of the initial lag-phase was found to increase with dose. Part of this repopulation delay may be attributed to radiation-induced division delay (22, 31–35).

However, little is known about possible changes of the kinetics of repopulation by clonogenic tumour cells occurring in the course of fractionated treatment. There are some experimental indications (36–38) that repopulation after single doses might be different from repopulation during fractionated irradiation, at least in our tumour system.

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The aim of the present paper was to study the repopulation of the tumour by clonogenic tumour cells during treatment and its influence on the response of the rhabdomyosarcoma R1H of the rat to fractionated radiotherapy. For this, a constant number of 30 fractions of x-rays was applied in different overall treatment times ranging from 10, 18.5, 29, 39, to 67 days. The approach is complicated by the fact that the rate of repopulation cannot be measured directly, mainly because of the fact that it is overlaid and modified by a great number of other parameters contributing to the overall effect. This requires assessment of tumour response by different experimental parameters such as net growth delay and in vitro cell survival.

Material and Methods

Tumour-host system. The experiments were performed on the rhabdomyosarcoma R1H of the rat. This transplantable solid tumour, which grows with a doubling time of 3 days, was derived from the R-1 (3) which originates from the BA 1112 tumour (39). The tumour shows no signs of specific immunogenicity (40). Transplantation was performed by implanting a piece of tumour tissue (about 1 mm^3 in size) subcutaneously into the back (laterally, subcostally) of male WAG/Rij albino rats (200–220 g) purchased from the TNO-REP Institutes, Rijswijk, The Netherlands. The animals were provided with food and water ad libitum. Further details of the tumour-host system were communicated previously (29, 37).

Irradiation. Tumours were locally irradiated with 200 kVp x-rays (0.5 mm additional Cu filtering; dose rate 1.9 Gy/min). Fig. 1 shows a scheme of the various fractionation regimens used in this study. All treatments consisted of 30 fractions. Various doses per fraction were applied during the different overall treatment times (10 days: 1.67, 1.90, 2.13, and 2.37 Gy/fraction; 18.5 and 39 days: 1.50, 2.00, and 2.50 Gy/fraction; 29 and 67 days: 2.00, 2.50, and 2.67 Gy/fraction). As anesthesia is known to change blood flow and temperature of animals and tumours (41, 42) and might even change their radiosensitivity (43), unanesthetized rats were used in order to avoid uncontrollable irradiation conditions. The animals were constrained in a jig (37), which allowed treatment of the tumours without considerable irradiation of lungs, spinal cord, and gut. However, one kidney was generally included within the irradiation field. The correct positioning of the tumour during irradiation was monitored using a TV-camera. In order to obtain homogeneous dose distribution, the tumours were irradiated from alternating sides from day to day. The tumours were treated under ambient conditions, i.e. without clamping.

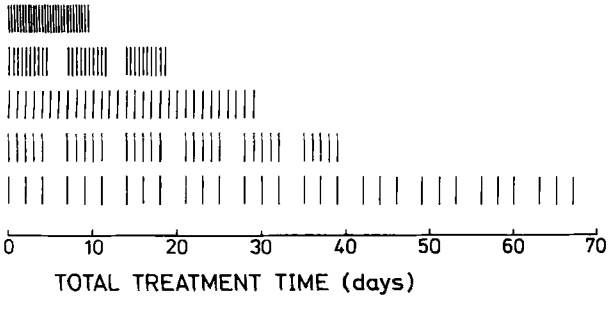

Fig. 1. Schematic presentation of the various fractionation schedules applied.

Tumour volume. Tumour volume was determined by measuring the tumours in two perpendicular dimensions by vernier calipers and assuming the shape to be a rotational ellipsoid. Volume was calculated after correcting for skin thickness. For reasons of convenience, tumours (approximately 6 per dose group per fractionation scheme) were included into the experiment only once a week. The minimal volume for inclusion was 0.24 cm^3. Due to tumour growth, the maximal volume was 3.7 cm^3. Thus, the range of variation of the tumour volumes at start of treatment was about 1 order of magnitude. The large range of tumour volumes has only a negligible influence on the tumour net growth delay (44). Animals were killed for ethical reasons when tumour volume exceeded 17 cm^3.

Net growth delay. The tumour volume of individual tumours was measured twice a week and from the resulting growth curves the net growth delay was derived. Net growth delay is the time interval between the time at which the tumour reaches twice its minimal volume after passing the nadir and the time at which the tumour has the same volume before irradiation (45). Using the net growth delay has the advantage of preferentially measuring the effects on the clonogenic tumour cells, whereas the influence of the tumour bed effect is minimized.

Colony assay. The number of clonogenic tumour cells per tumour was determined at different intervals during fractionation treatment by means of an in vitro colony assay. Animals were asphyxiated in CO_2, tumours removed and weighed and then single cell suspensions were prepared by trypsinization. A yield of 2.8×10^6 tumour cells/g of tissue was obtained for untreated tumours. The cells were subsequently plated in plastic Petri dishes carefully discriminating tumour and host cells and the clonogenic fraction was determined. The mean plating efficiency of control tumours was 63%. For further details of the experimental procedure, see ref. 37.

Results

Fig. 2 shows the growth curves obtained after treatment with 30 fractions of 2 Gy in 10, 39, and 67 days and the growth curves obtained for untreated tumours. By the end of the 10 days schedule, the tumours just began to shrink but still kept nearly their start volume; these tumours continued to shrink after the end of treatment for about 1

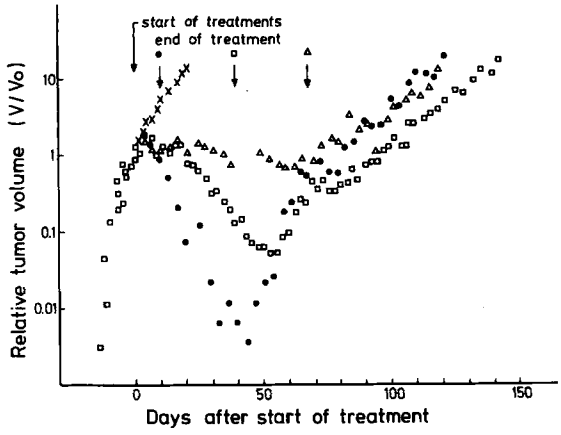

Fig. 2. Growth curves obtained for R1H rhabdomyosarcoma of the rat after delivering 30 fractions of 2 Gy in (●) 10 days, (□) 39 days, or (△) 67 days. The figure shows the data of the individual tumours with the median net growth delay. Locally controlled tumours were included in the calculation of the net growth delay.

month. When the overall treatment time was extended to 39 days, a considerable portion of the shrinking process was already completed by the end of irradiation; the tumours continued to shrink for another 2 weeks. After 67 days of treatment, the tumours regrew immediately after the end of irradiation. While the tumour volume was reduced by more than two orders of magnitude after 60 Gy administered in 10 days, this reduction was only about one order of magnitude for the standard overall treatment time of 6 weeks and nearly no tumour shrinkage was observed when irradiation was extended to 67 days.

In Fig. 3 the influence of the overall treatment time on net growth delay is depicted. Such a comparison is only meaningful for treatment schedules where the same number of fractions and the same dose per fraction is given in different overall treatment times. The data plotted in this figure were obtained for 30 fractions of 2 Gy each (total dose of 60 Gy) given in 10 to 67 days. The net growth delay increased for overall treatment times between 10 and 18.5 days and remained more or less constant for treatment times lasting longer than 3 weeks indicating a change in repopulation kinetics at this time.

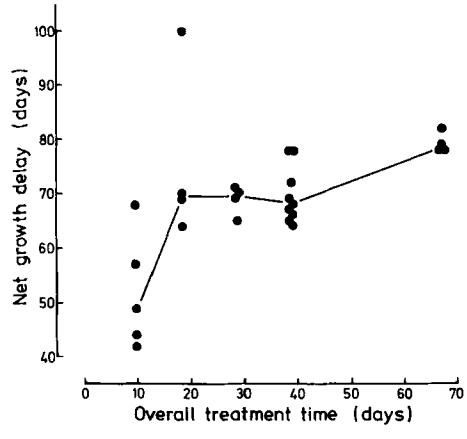

Fig. 3. Net growth delay as obtained after fractionated irradiation with 30 fractions of 2 Gy is shown as a function of overall treatment time. The data of the 10-day group were interpolated from results derived from regimens of 30 fractions of 1.90 and 2.13 Gy.

Discussion

Fig. 2 shows the growth curves of tumours from different treatment schedules. At an overall treatment time of 67 days, repopulation seems to be sufficient to balance the cell kill achieved by the 30 fractions of 2 Gy, resulting in a net growth delay of 78 days (Fig. 3). Assuming a constant inactivation per fraction with a D_0 of 4.3 Gy (37), an averge doubling time of about 4 days is derived over the whole treatment period, which is somewhat slower than in unirradiated control tumours (3 days).

The evaluation of experimental data on tumour growth delay is complicated by a number of counteracting mechanisms and may result in misleading conclusions. The problems arise when schedules differing in overall treatment time are to be compared. Growth delay should be independent of the overall treatment time (46) provided the inactivation per fraction is constant and identical for the schedules compared, as is demonstrated schematically in Fig. 4A. However, this is only valid if the repopulation rate remains unchanged before, during and after treatment for all fractionation regimens concerned, since repopulation is an inherent part of the growth delay assay (46). Fig. 4B illustrates the situation which is to be envisaged when a complete cessation of repopulation occurs during treatment. In this case, the treatment with the longer overall treatment time leads to a longer growth delay, i.e. $GD_1 < GD_2$, although survival of tumour cells at the end of treatment is the same for both schedules compared. If the delay is assessed from the point of time when the last fraction is given instead of relating growth delay to the start of treatment, differences in growth delay more closely reflect repopulation between fractions. Therefore, the results obtained when the overall treatment time T is subtracted from the growth delays are similar for both treatments, that is $GD_1 - T_1 = GD_2 - T_2$. Fig. 4C shows an intermediate situation between examples A and B. The repopulation rate is constant during treatment but lower than in control tumours. In this case, we obtain: $GD_1 < GD_2$ and $GD_1 - T_1 > GD_2 - T_2$.

If net growth delay was assessed from the time of the last fraction (nGD − T) and plotted as a function of overall treatment time (Fig. 5), it was approximately constant for overall treatment times of up to 20 to 30 days. This indicates that repopulation is very small during treatments of less than 20 to 30 days. For longer treatment

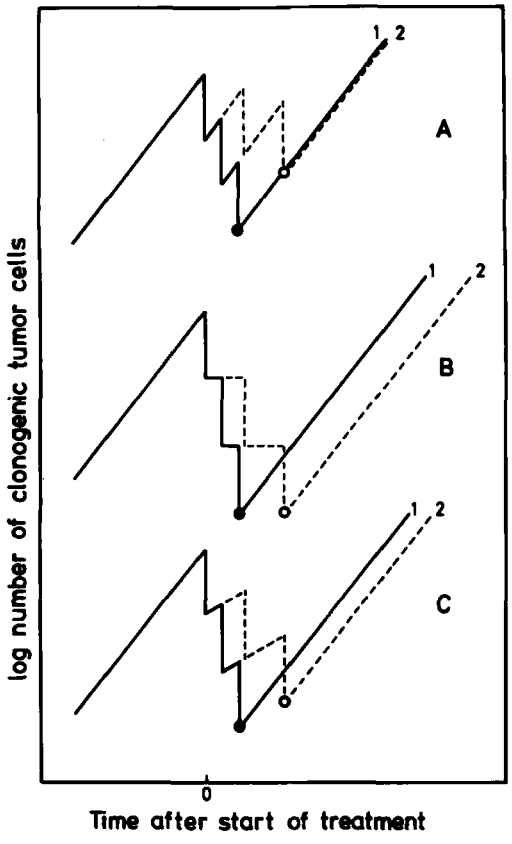

Fig. 4. Schematic presentation of the relationship between number of clonogenic tumour cells, repopulation, growth delay (GD), and overall treatment time (T), when different treatments are compared. In all charts the number of clonogenic tumour cells per tumour is plotted versus treatment time. A) undisturbed repopulation during treatment; $GD_1 = GD_2$. B) complete cessation of repopulation during treatment; $GD_1 - T_1 = GD_2 - T_2$. C) intermediate situation between undisturbed and no repopulation; $GD_1 < GD_2$ and $GD_1 - T_1 > GD_2 - T_2$. (●) and (○) denote number of clonogenic tumour cells at the end of the two fractionated treatments compared.

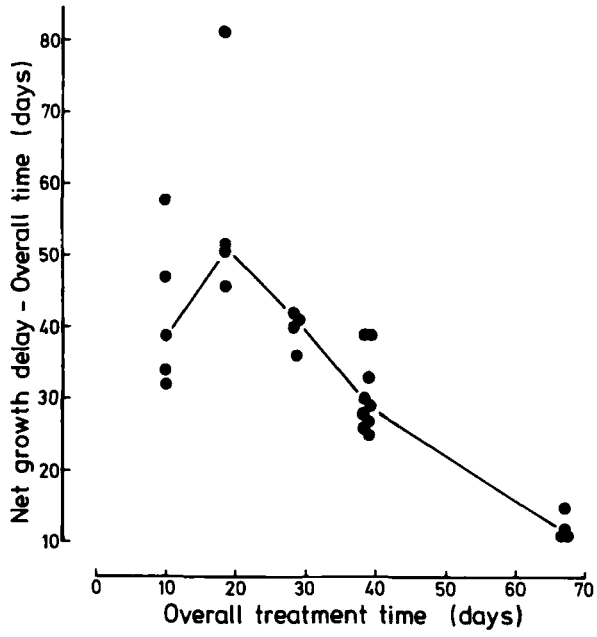

Fig. 5. Net growth delay after subtraction of overall treatment time as obtained after fractionated irradiation with 30 fractions of 2 Gy is shown as a function of overall treatment time. The data of the 10 day group were interpolated from results of the regimens of 30 fractions of 1.90 and 2.13 Gy.

periods net growth delay decreased, whereby the rate of decrease corresponded to a doubling time of repopulation tumour cells of about 4.5 days at that time. An overall treatment time of 81 days should lead to a net growth delay that is equal to the overall treatment time. That means keeping tumour volume approximately constant during treatment when 30 fractions of 2 Gy are delivered in 81 days.

Information about the inactivation kinetics of the clonogenic tumour cells within a tumour during fractionated irradiation treatment can be obtained by the colony assay. However, it has to be considered that the changes in number of clonogenic tumour cells during fractionated irradiation are determined by two processes: inactivation during irradiation and repopulation in the interval between the fractions. Repopulation comprises production of clonogenic cells minus cell loss.

To estimate the influence of repopulation separately, the influence of inactivation has to be calculated and subtracted from the results. In a previous study (37) the kinetics of cellular inactivation by fractionated irradiation in the rhabdomyosarcoma R1H of the rat was determined in the dose range of 1.07 to 12.50 Gy (median: 2.50 Gy) per fraction. Regimens of 1, 3, 5, 7, and 10 fractions per week for several weeks were compared. The number of clonogenic tumour cells per tumour in the course of the different treatment schedules was obtained using an in vitro colony assay. The results showed that the fraction of tumour cells inactivated per week was only dependent on the total dose per week, i.e. the cellular response was the same whether the weekly dose was applied in 1, 3, 5, 7, or 10 fractions. All fractionation regimens could be fitted with a D_0 of 4.3 ± 0.2 Gy. These findings were confirmed by other studies (38, 47), where tumour response was assessed by tumour control probability and net growth delay. Setting out from these data, the relative repopulation during fractionated treatment can be determined by calculating the number of clonogenic tumour cells per tumour corrected for inactivation N'(t) using the equation:

$$N'(t) = N_c(t, D) \cdot \exp(D/D_0)$$

where D represents the total dose up to time t and $N_c(t, D)$ the number of clonogenic tumour cells per tumour as determined by colony assay.

Figure 6 shows the results obtained by reevaluation of the data reported earlier (37) using the equation given above. Data from various fractionation schemes (1 to 10

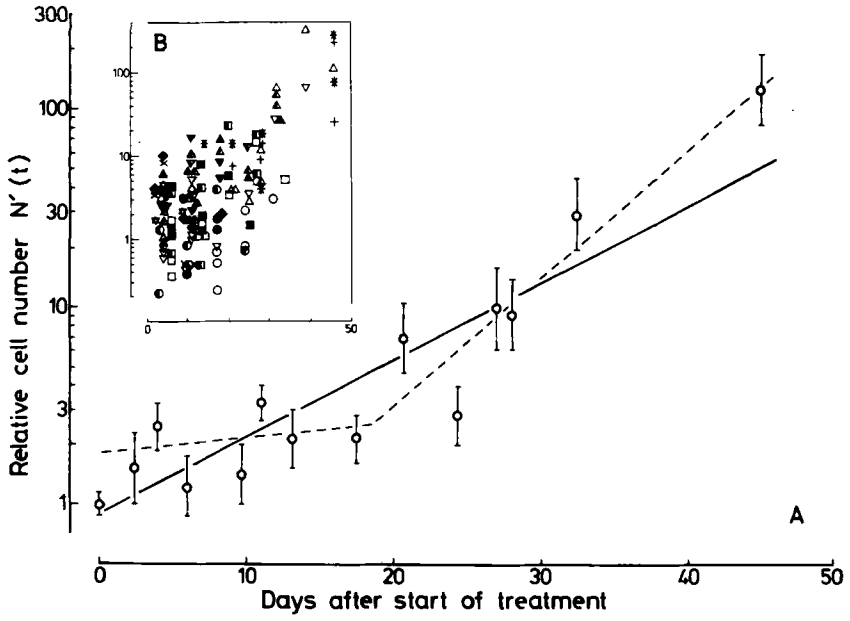

Fig. 6. Relative repopulation during fractionated treatment. The number of clonogenic tumour cells per tumour corrected for inactivation $[N'(t) = N_c(t, D)\exp(D/D_0)]$ is plotted versus time after start of treatment. A) Pooled data taken from ref. 37. A linear regression yields a slope that corresponds to a doubling time of 8 ± 1 days (solid line). The results could also be fitted by a two-component linear regression with an initial slope corresponding to virtually no repopulation up to day 20 and a second slope corresponding to a doubling time of 5 ± 1 days for treatment times exceeding 20 days (broken line). B) Same data as A) but identifying the different treatment schedules. The doses per week for the 1-fraction/week regimen were: (○) 7.5 Gy, (◐) 10 Gy, (●) 12.5 Gy; 3 fractions/week: (+) 6 Gy, (△) 7.5 Gy, (*) 8 Gy, (◭) 10 Gy, (▲) 12.5 Gy; 5 fractions/week: (▽) 7.5 Gy, (◬) 10 Gy, (▼) 12.5 Gy; 7 fractions/week: (□) 7.5 Gy, (◧) 10 Gy, (■) 12.5 Gy; 10 fractions/week: (◆) 15 Gy, (×) 20 Gy, (✡) 25 Gy.

fractions per week) are shown. A linear regression yields a slope that corresponds to a doubling time of 8 ± 1 days, indicating that repopulation is decelerated during treatment (volume doubling time of unirradiated tumours: 3 days). The slope is equivalent to a repopulated dose of 0.37 Gy per day (0.33–0.43 Gy/d). However, the linear regression does not seem to fit the data appropriately. There seem to be systematic deviations pointing to an overestimation in the range of overall treatment times of 5 to 30 days, and an underestimation for overall treatment times below 5 and above 30 days. The results appear to be more adequately fitted by a two-component linear regression with a slope corresponding to virtually no growth up to day 20 and a slope corresponding to a doubling time of 5 ± 1 days or a repopulated dose of 0.60 Gy per day (0.50–0.75 Gy/d) for overall treatment times exceeding 20 days. This still represents a decelerated repopulation as compared to unirradiated control tumours.

The statistical accuracy does not allow decision concerning which of the two interpretations is most adequate. The analysis shows that it is very difficult to obtain reliable data on the repopulation kinetics of the surviving tumour cells during fractionated treatment with the excision assay (48). Nevertheless, the colony assay data seem to support the biphasic interpretation of the results derived from growth delay measurements. The results suggested that repopulation during treatment began about 3 weeks after start of irradiation in our tumour system. These observations are in line with data reported on C3H mammary carcinoma showing that for longer treatment schedules repopulation was the governing factor that impeded local tumour control (49).

Our results are at variance with the unexpectedly fast repopulation during fractionated irradiation of a slow growing tumour reported by Kummermehr & Trott (5). However, in this study, tumours were artificially clamped during irradiation to render all cells hypoxic. Clamping of tumours has been shown to have a considerable effect on the influence of overall treatment time on tumour response to fractionated irradiation. In a comprehensive study with the C3H mouse mammary carcinoma, Suit et al. (8) investigated the relationship between total radiation dose required to control 50% of the tumours locally ($TCD_{50\%}$) and overall treatment time. Radiation was administered to anaesthetized mice under conditions of acute local hypoxia produced by clamping, normal air breathing and respiration of 100% oxygen under hyperbaric conditions. Under clamped conditions the $TCD_{50\%}$ after application of 10 fractions increased with increasing overall treatment time. In contrast, the $TCD_{50\%}$ remained constant under ambient and hyperbaric oxygen conditions for the entire range of treatment times studied. The influence of clamping on tumour response is discussed in detail elsewhere (50).

There is a good correlation between our experimental

data and clinical findings. Trott & Kummermehr (23) reanalyzed data from Friedmann et al. (10) on Hodgkin's disease and found a significant increase of the tumour control dose $TCD_{50\%}$ for overall treatment times longer than 3 weeks. For these treatment times the authors calculated a repopulated dose of 0.30 Gy per day. Local control rates of larynx carcinoma were found to depend strongly on overall treatment time, especially between 4 and 7 weeks (17). During that period a repopulated dose of 0.5 Gy per day was calculated, corresponding to a doubling time of clonogenic cells of 4 days. Our findings are, furthermore, in excellent agreement with data recently reported by Withers et al. (19) who estimated the doses to achieve local control in 50% of cases from published local control rates for squamous cell carcinoma of head and neck, and the dependence of these doses on overall treatment duration was evaluated. In parallel, published scattergrams were analyzed to estimate the rate of tumour regrowth over the period of 4 to 10 weeks from the onset of therapy. Both analyses suggested that, on average, clonogen repopulation accelerates only after a lag period of the order of 4 ± 1 weeks after initiation of radiotherapy.

Many of the preceding calculations and deductions are based on the assumption of a constant inactivation per fraction. The opposite approach is to assume a constant doubling time of the clonogenic tumour cells during fractionated irradiation. Analogue calculations lead to the conclusion that the inactivation per fraction decreases during the course of a fractionated radiotherapy and that in our tumour system reoxygenation is continously reduced after about 3 weeks after start of treatment. This, however, seems to be very improbable, since it coincides with the time at which the tumours begin to shrink. An increased repopulation after 3 weeks of treatment thus appears to be much more probable.

The results presented in this paper reveal that accelerated repopulation, as has been reported for several experimental tumours after single dose treatment, does not apply to fractionated treatment. During fractionation the repopulation of clonogenic tumour cells seems to be considerably slowed down in the rhabdomyosarcoma R1H, especially during the first 3 weeks of treatment.

It is concluded that in treatments lasting less than 3 weeks, the repopulation of the tumour by clonogenic tumour cells is only of minor importance, whereas for periods exceeding 3 weeks, its influence becomes increasingly decisive for treatment outcome of a fractionated radiotherapy as far as the response of the rat rhabdomyosarcoma R1H is concerned.

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