Europ. J. Cancer, Vol. 17, No. 4, pp. 375–386, 1981
Printed in Great Britain

0014–2964/81/040375-12 $02.00/0

Depopulation and Repopulation of the R1H Rhabdomyosarcoma of the Rat after X-Irradiation

H. JUNG, H.-P. BECK, I. BRAMMER and F. ZYWIETZ

Institute of Biophysics and Radiobiology, University of Hamburg, Martinistraße 52, 2000 *Hamburg* 20, *Federal Republic of Germany*

Abstract—*Experiments were carried out to study the kinetics of depopulation and repopulation of the solid transplantable rhabdomyosarcoma R1H of the rat following local irradiation with a single X-ray dose of* 15 Gy. *Several parameters were sequentially measured over a time interval of* 25 days *after irradiation: The ratio of tumour to host cells was determined by flow cytometry; the numerical density of tumour cells was obtained by stereological analysis of histological slides; the clonogenic fraction of tumour cells was assayed by plating an appropriate number of tumour cells and scoring the colonies; tumour volume was assessed by measuring two tumour diameters at right angles to each other. All parameters investigated, except tumour volume, undergo drastic changes during the first* 2 weeks *after irradiation. From the directly measured parameters the following values and their variation with time could be derived. The number of host cells per tumour increased by a factor of* 10 *within the first* 10 days *after irradiation, probably due to infiltration by blood-borne host cells. During the same time interval, the number of tumour cells decreased by a factor of* 3, *whereas the total number of cells per tumour showed an increase by a factor of* 4. *Since the host cells are considerably smaller than the tumour cells, the cellular numerical density increased by a factor of* 3, *but approached the control level by* Day 18 *after irradiation. From the number of clonogenic and non-clonogenic tumour cells the kinetics of repopulation and depopulation was obtained. Repopulation of irradiated tumours by surviving tumour cells as well as removal of inactivated tumour cells began immediately after irradiation and proceeded with exponential kinetics. Repopulation occurred with a doubling time of* 4.4 ± 0.3 days *whereas inactivated tumour cells disintegrated with a halving time of* 3.5 ± 0.7 days. *There were no indications that proliferation of doomed cells contributed significantly to tumour growth after X-irradiation.*

INTRODUCTION

FOLLOWING subcurative radiation treatment, solid tumours usually undergo typical volume responses. After exposure to moderate radiation doses, tumours may continue to increase in size for a few days after treatment. Since at the same time, the fraction of clonogenic tumour cells is reduced by one to three orders of magnitude, there exists a large discrepancy between volume response and surviving tumour cells. Later on, the tumours begin to shrink as the tumour cells that have been sterilized as a result of treatment lyse and are resorbed. Concurrent with these events, surviving tumour cells begin to proliferate and repopulate the tumour, resulting in volume regrowth. Thus, it is the competing processes of resorption and repopulation that are the major factors determining the volume response curve of a tumour [1].

In general, changes in tumour volume after irradiation reflect rather poorly changes in the numbers of remaining cells [2] as well as changes in the size of the clonogenic cell population [3, 4]. The development of experimental tumours for which the fraction of clonogenic cells can be assayed *in vitro* has made it possible to study the kinetics of repopulation of these tumours after irradiation (cf. [5, 6]) or after chemotherapy (cf. [7]). But many questions concerning basic factors of repopulation or the applicability of certain experimental procedures are still far from being solved. Much less information is available, at present, about the rate of clearance of dead

Accepted 21 October 1980.

cells from irradiated solid tumours. The reason is that most methods that have been developed for the kinetic analysis of tumours [8] are not applicable to tumours that have been treated with radiation or with chemotherapeutic drugs.

In this paper, we describe experiments designed to study the kinetics of depopulation and repopulation of a rhabdomyosarcoma of the rat following local irradiation with a single X-ray dose of 15 Gy. By measuring the ratio of tumour to host cells, the numerical density of tumour cells, the clonogenic fraction of tumour cells and the tumour volume as a function of time interval after irradiation it was possible to derive the number of tumour cells and host cells per tumour as well as the number of clonogenic and non-clonogenic tumour cells. The rationale of our approach was to describe the cell kinetic changes on the basis of cell numbers per tumour, thus eliminating the contribution of necrotic and fibrotic regions, since they do not primarily affect the cell kinetic behaviour of the considered population, although their contribution to tumour volume might be considerable.

MATERIALS AND METHODS

Tumour

The experiments were performed on the rhabdomyosarcoma R1H (H = Hamburg) of the rat. This transplantable solid tumour was derived from the rhabdomyosarcoma R-1 described by Barendsen and Broerse [3]. The R-1 tumour was kindly provided by Prof. G. W. Barendsen in 1976 and has been maintained since then in our laboratory by serial transplantation on inbred WAG/Rij rats. Within two years, the DNA content of the tumour cells increased from 1.4 [9] to 4.3 times that of host cells but has been constant for the last two years. Parallel with the increase in DNA content, the volume of the tumour cells increased. These changes have proved to be highly advantageous because the tumour cells may be distinguished clearly from host cells not only in the flow cytometer, but also on histological slides and when cell suspensions are counted in a counting chamber. Tumour take, determined after injecting 50 tumour cells per animal, was found to be 100% (20/20).

Transplantation

Transplantation of the R1H tumours was performed by implanting a piece of tumour tissue, about 1 mm^3 in size, subcutaneously into the right flank of male WAG/Rij albino rats (200–220 g) purchased from the TNO-REP institutes, Rijswijk, The Netherlands. The animals were kept under a 12 hr light, 12 hr dark regimen and provided with food and water *ad libitum*.

Tumour volume

Tumour volume was determined by measuring the tumours prior to irradiation and excision in two perpendicular dimensions, using vernier calipers. Tumour volume was calculated assuming the shape to be a rotational ellipsoid. The standard deviation (S.D.) was estimated to amount to $\pm 20\%$.

Irradiation

Twenty days after transplantation, a series of tumours weighing 1.6 ± 0.4 g ($\pm$S.D.) were locally irradiated with 15 Gy of 200 kVp X-rays (0.5 mm Cu filtering; lead collimator 30 mm in dia.; dose rate 1 Gy/min). For exposure, the rats were anaesthetized with sodium pentobarbitone (Nembutal), injected intraperitoneally at 60 mg per kg body wt 10 min before irradiation. At various times after irradiation, tumours were excised and cut into slices of 3 mm thickness so that several parameters could be determined for each tumour.

Flow cytometry

Flow cytometric DNA measurements were performed after preparing single cell suspensions. In order to take into account the heterogeneity in the spatial distribution of tumour cells, tissue was taken from the centre and from the periphery of each tumour slice and investigated separately [10]. The tissue was minced and suspended in 5 ml phosphate-buffered saline. The suspension was syringed a few times and 10 ml of a 0.1% pepsin solution (dissolved in 0.2% HCl) was added. After stirring for 5 min, the suspension was filtered through 53 μm nylon mesh and 1 ml of the filtrate was stained in the dark for 10 min with 15 ml of a fluorescent dye solution containing 5 μg/ml ethidium bromide and 12.5 μg/ml mithramycin. The flow cytometric measurements were performed using an ICP-22 detector (Phywe, Göttingen) equipped with a high pressure mercury lamp, excitation filters BG3, BG38 and KG1 and a barrier filter OG580. The instrument was calibrated

with spleen cells from the host animal. The histograms were evaluated with a computer program correcting for background [11]. The proportion of tumour cells (i.e., the ratio of tumour to tumour plus host cells) was determined from the integrals of the peaks of the tumour and host cell population. The volume-weighted mean of the data obtained for the centre and the periphery of each tumour was calculated yielding a value representative of the whole tumour. The standard deviation of the results was estimated to amount to $\pm 10\%$ as determined from numerous serial FCM studies using the same experimental procedure.

Morphometry

The central slice of each tumour was subjected to routine histological procedures including formalin fixation, paraffin embedding, sectioning at 3 μm and staining with hematoxylin and eosin. The microscopic slides were analysed according to stereological principles [12] in order to determine the number of tumour cell nuclei per unit of volume (i.e., the numerical density of tumour cells). This parameter was obtained by measuring the volumetric density as well as the average volume of tumour cell nuclei. Tumour cell nuclei were distinguished from host cell nuclei on the basis of their size and morphological appearance.

The volumetric density (i.e. the fraction of volume occupied by tumour cell nuclei) was determined by the differential point counting method [13]. Using a pattern of 10 test points per microscopic field, the frequency of hits on tumour cell nuclei was determined. After correcting for finite section thickness [14], the volumetric density of tumour cell nuclei was obtained. Radial variations in the distribution of tumour cells were taken into account by grouping the data of about 400 fields per section according to stratified sampling [15], the strata representing equidistant shells. The volume-weighted mean was taken as an estimate of the tumour as a whole.

In order to correct for finite section thickness as well as to convert volumetric density (volume per volume) into numerical density (number per volume), the average volume of the nuclei was determined by measuring the length of the linear intercepts of tumour cell nuclei with a given array of parallel scanning lines. From the mean of linear intercepts ($N=300$), the mean diameter of the nuclei was calculated [16] and converted to volume assuming the nuclei to be spherical and of equal size. The errors indicated represent standard deviations (S.D.) determined from the variation between different microscopic fields and propagating the errors to the final results.

The proportion of host cells was determined morphometrically at Day 8 to amount to 87 $\pm 5\%$ which, within experimental error, agrees with the value measured by flow cytometry ($95 \pm 9\%$), thus indicating that the cell suspension prepared for flow cytometric measurements is representative of the ratio in intact tumour tissue. The actual cellularity of untreated tumours, as determined by morphometry, amounted to 3×10^8 tumour cells per cm^3 of tumour tissue using a volume shrinkage factor of 0.6. Further details of these lengthy and time-consuming procedures as well as additional results will be published elsewhere.

Assay of clonogenic tumour cells

Another piece of each tumour was used to prepare a cell suspension according to the dispersion technique described by Reinhold [17]. The tissue was minced with two crossed scalpels and then stirred in 150 ml of a 0.1% trypsin solution in Hanks buffer for 15 min by means of a rotating razor blade. The cells were sedimented at 1000 ***g*** for 10 min and resuspended in 10 ml Eagle's medium. After filtering through a four-fold layer of sterile gauze, the number of tumour cells per ml was counted in a Fuchs–Rosenthal counting chamber. An appropriate number of tumour cells (disregarding host cells) were plated into Falcon plastic petri dishes, 50 mm in diameter, which contained 5 ml of Eagle's medium, supplemented with 15% fetal calf serum and bykomycin (Byk Gulden, Konstanz, Germany) as well as R1H feeder cells, irradiated with 40 Gy, to make a total number of 10^4 cells per dish. After incubation for 10 days, colonies were fixed, stained and counted using a projection device described earlier [18]. Since the host cells do not form colonies under the experimental conditions applied, the procedure described yields the fraction of tumour cells that are clonogenic. Plating efficiency of the controls amounted to $47.1 \pm 3.4\%$. The errors indicated represent standard deviations calculated from the statistical variations ($N \pm \sqrt{N}$) of the number of tumour cells counted in the chamber, the number of cells plated and the number of colonies scored.

RESULTS

Measured parameters

Figure 1 shows two flow cytometric histograms of R1H tumours. In an unirradiated control recorded at Day 0 (chart A), the G1 peak of the host-derived cells appears at a relative fluorescence intensity of 21. The corresponding G2 cells appear at channel 42, whereas the tumour parenchymal cells are

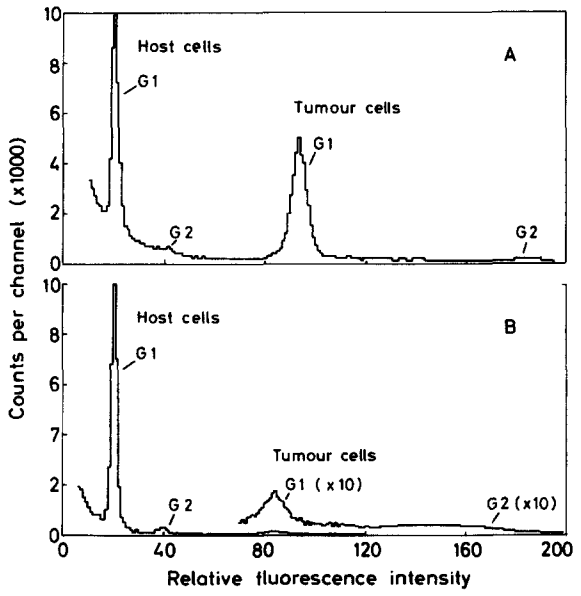

Fig. 1. Flow cytometric histograms of the rhabdomyosarcoma R1H of the rat, recorded using a flow cytometer ICP-22. Tissue was taken from tumour periphery to prepare a cell suspension by mechanical shearing and gentle pepsin treatment followed by staining with a mixture of ethidium bromide and mithramycin. Chart A: Histogram of control. Chart B: Histogram recorded at Day 8 *after irradiation with* 15 Gy *of X-rays.*

recorded at channels 93 (G1 cells) and 185 (G2 cells). The cell suspension investigated consists of 55% tumour cells and 45% host cells. Figure 1 (chart B) shows a histogram of an R1H tumour irradiated with 15 Gy and recorded at Day 8 after irradiation. Only 5.3% of the cells are tumour cells indicating that the proportion of tumour cells decreases by about one order of magnitude after irradiation as compared to the untreated control (chart A). The proportion of tumour cells was determined over a period of 25 days after irradiation. The results obtained are displayed in Fig. 3 [curve $P_T(t)$].

Figure 2 (chart A) shows the volumetric density of tumour cell nuclei as determined by differential point counting. In the unirradiated controls and immediately after irradiation, $18.4 \pm 1.3\%$ of total tumour volume consists of tumour cell nuclei. This fraction

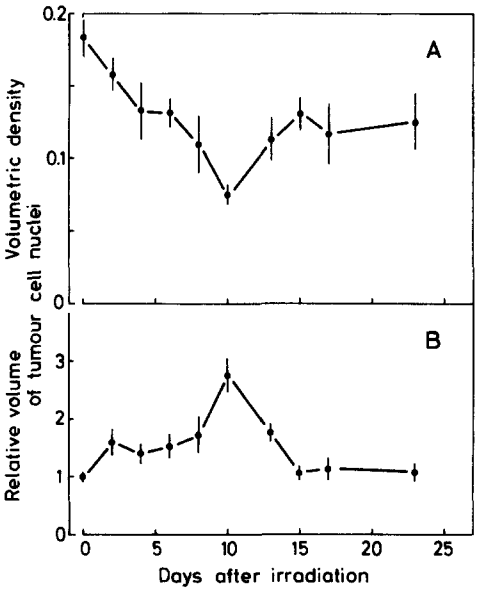

Fig. 2. Morphometric changes of tumour cell nuclei occurring in the R1H rhabdomyosarcoma after irradiation with 15 Gy *of X-rays. Chart A: Volumetric density (i.e. the fraction of volume occupied by tumour cell nuclei) as determined by differential point counting on microscopic slides ($N \approx 4.000$ per tumour). Chart B: Relative volume of tumour cell nuclei determined by measuring the length of the linear intercepts of tumour cell nuclei with a given array of parallel scanning lines ($N = 300$ per tumour). Error bars represent standard deviations (S.D.).*

decreases to $7.5 \pm 0.7\%$ during the first 10 days after irradiation, and increases afterwards to a constant level amounting to about 70% of the control value.

The volume of tumour cell nuclei (Fig. 2, chart B), as determined by the method of linear intercepts, increases by a factor of 2.8 ± 0.3, decreases afterwards and approaches the control level at Day 15. From the two curves displayed in Fig. 2, the numerical density of tumour cells, $D_T(t)$ may be determined. This parameter, which is the ratio of volumetric density and nuclear volume, is plotted in Fig. 3 as a function of time after irradiation [curve $D_T(t)$].

Figure 3 shows the parameters which were directly measured for each individual tumour, as a function of time interval after irradiation with 15 Gy. Besides the data described above [i.e. the proportion of tumour cells $P_T(t)$, and the numerical density of tumour cells, $D_T(t)$], the tumour volume, $V(t)$, and the clonogenic fraction of tumour cells, $C_T(t)$, are plotted. Since tumour volume, $V(t)$, was determined for single tumours, scatter of the data is considerable. After irradiation with 15 Gy, tumour growth is slowed down for about 2 weeks, followed by an increase in growth rate. By Day 22, growth rate of irradiated tumours approaches that of control tumours of comparable size [10].

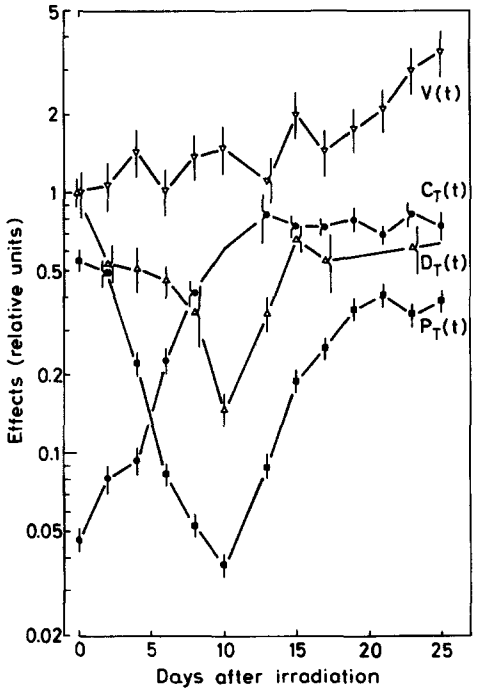

Fig. 3. Changes of several parameters occurring in the R1H rhabdomyosarcoma of the rat after irradiation with 15 Gy *of X-rays. Each set of data has been determined for the same tumour. Data were normalized to the values measured for control tumours at the time of irradiation. Error bars represent standard deviations (S.D.). Curve* V(t): *Relative volume of the investigated tumours determined by caliper measurements at the time of irradiation and of excision. Curve* $D_T(t)$: *Numerical density of tumour cells determined as the ratio of volumetric cell density (Fig.* 2A) *and nuclear volume (Fig.* 2B). *Curve* $P_T(t)$: *Proportion of tumour cells (i.e. ratio of the number of tumour cells to the number of all cells) as determined by flow cytometry. Experimental procedure as given in legend to Fig.* 1. *Curve* $C_T(t)$: *Clonogenic fraction of tumour cells. A given number of tumour cells determined in a counting chamber were plated in plastic petri dishes together with feeder cells (irradiated with* 40 Gy) *to make a total number of* 10,000. *Colonies were scored* 10 days *later after fixation and staining with crystal-violet.*

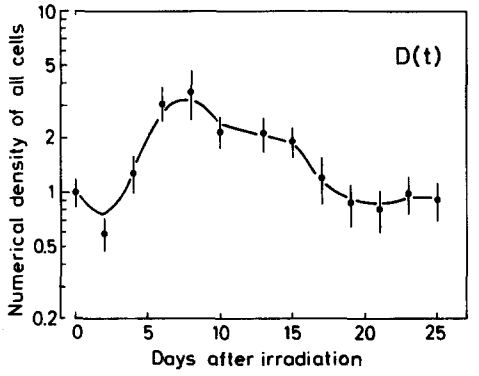

Fig. 4. Numerical density of all cells (i.e. number of all cells per unit of volume of tumour tissue; in relative units) determined for the R1H rhabdomyosarcoma as a function of time interval after irradiation with 15 Gy *of X-rays. The data plotted have been obtained from the measured values (Fig.* 3) *according to equation* (1). *Error bars represent standard deviations (S.D.). The curve drawn has been smoothed by averaging the values over nearest neighbouring points weighted by* 0.2.

Immediately after a dose of 15 Gy, survival of tumour cells is reduced to $4.6 \pm 0.5\%$ [Fig. 3, curve $C_T(t)$]. With increasing time interval after irradiation, the clonogenic fraction of the tumour cells increases steadily and reaches, at Day 13, a constant level amounting to about 80% of unirradiated tumours at Day 0.

From the data shown in Fig. 3 it is obvious that all the parameters investigated undergo pronounced changes during the first 2 weeks after irradiation. It should be emphasized that the tumour volume, the parameter most frequently used to quantify the response of tumours to irradiation, shows the smallest changes of all the parameters measured.

Derived parameters

From the data plotted in Fig. 3, a number of further parameters ($\pm$S.D. determined according to propagation of errors of independent variables) and their variation with time may be derived.

Numerical density of all cells. Figure 4 shows the relative changes of the numerical cell density, $D(t)$ (i.e. the number of all cells per unit of volume), as a function of time interval. This quantity is obtained from the numerical density of tumour cells, $D_T(t)$, and the proportion of tumour cells among all cells, $P_T(t)$, normalized to the value at $t=0$, $P_T(0)$:

$$D(t)=\frac{D_T(t)}{P_T(t)}\cdot P_T(0). \qquad (1)$$

During the first 2 days after irradiation, the numerical density seems to decrease somewhat, perhaps due to a radiation-induced division delay accompanied by an increase in cellular volume. After Day 4, cell density increases, reaches a maximum of 3 times the normal density by Day 8, and returns to the control level at Day 18.

Number of cells per tumour. For cell kinetic considerations, cell numbers per tumour are more relevant parameters than numerical densities. From the data of Fig. 3, we may obtain for each tumour the number of all cells, $N(t)$, the number of tumour cells, $N_T(t)$, as well as the number of host cells, $N_H(t)$:

$$N(t)=D(t)\cdot V(t) \qquad (2)$$

$$N_T(t)=N(t)\cdot P_T(t)=D_T(t)\cdot P_T(0)\cdot V(t) \qquad (3)$$

$$N_H(t)=N(t)-N_T(t). \qquad (4)$$

The cell numbers determined according to equations (2)–(4) are shown in Fig. 5. Since

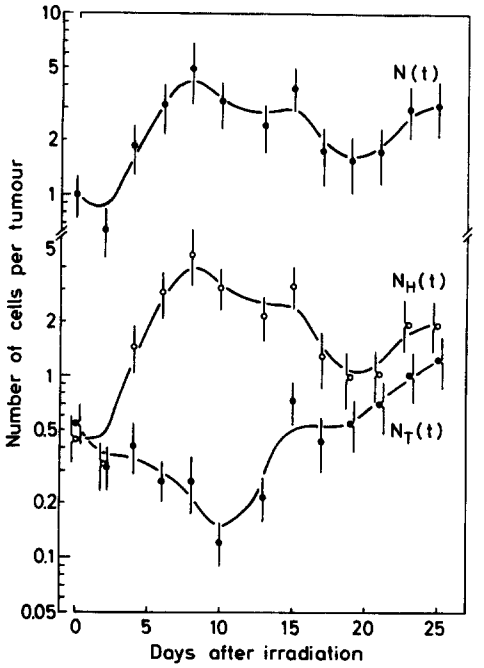

Fig. 5. Number of cells per tumour (in relative units) determined for the R1H rhabdomyosarcoma as a function of time interval after irradiation with 15 Gy *of X-rays. The data plotted have been obtained from the measured values (Fig. 3) according to equations* (2)–(4). *Error bars represent standard deviations (S.D.). The curves drawn have been smoothed by averaging the values over nearest neighbouring points weighted by* 0.2. *Curve* N(t): *All cells. Curve* $N_T(t)$: *Tumour cells. Curve* $N_H(t)$: *Host cells.*

they are obtained by multiplication with tumour volume (curve $V(t)$, Fig. 3), the cell numbers show larger scatter and larger error bars than the values of numerical cell density. Nevertheless, Fig. 5 clearly shows that the number of all cells, $N(t)$, increases by a factor of about 4 within 8 days after irradiation, decreases afterwards, and increases again after Day 20, when the irradiated tumours are regrowing.

Despite the large increase in the total number of cells, the number of tumour cells, $N_T(t)$, is reduced continuously after irradiation until Day 10, when a minimum is reached that is a factor of 5 lower than the control level. This curve already gives some indications about depopulation and repopulation of the irradiated tumours but does not yet allow a quantitative evaluation or the separation of these two processes (see following section).

The number of host cells per tumour [Fig. 5, curve $N_H(t)$] increases by a factor of 10 within 8 days, decreases during the following 10 days and increases again due to tumour growth. This large increase in the number of host cells is probably not caused by an enhanced proliferation of host cells within the tumour but rather should be attributed to an infiltration of the irradiated tumours by blood-borne cells (cf. Discussion).

Clonogenic and non-clonogenic tumour cells. Depopulation and repopulation of the irradiated tumours may be quantified by determining the number of clonogenic tumours cells, $N_{T,c}(t)$, and the number of non-clonogenic tumour cells, $N_{T,nc}(t)$. Both values may be derived from the number of tumour cells, $N_T(t)$, and the clonogenic fraction, $C_T(t)$:

$$N_{T,c}(t) = N_T(t) \cdot C_T(t) \quad (5)$$

$$N_{T,nc}(t) = N_T(t) - N_{T,c}(t). \quad (6)$$

The data determined according to equations (5) and (6) are shown in Fig. 6. Immediately after irradiation, $2.5 \pm 0.7\%$ of all cells present in the tumour are clonogenic tumour cells; their number increases exponentially with

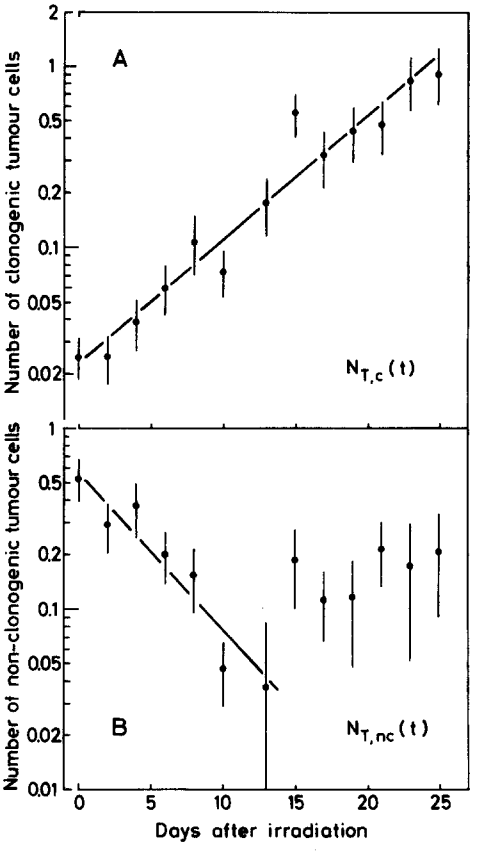

Fig. 6. Number of clonogenic tumour cells (chart A) and number of non-clonogenic tumour cells (chart B), in relative units, determined for the R1H rhabdomyosarcoma as a function of time interval after irradiation with 15 Gy *of X-rays. The data plotted have been obtained from the measured values (Fig. 3) according to equations* (5) *and* (6). *Error bars represent standard deviations (S.D.). From the straight lines drawn, calculated by least-squares analysis, the doubling time of clonogenic tumours cells repopulating the tumours was determined as* 4.4 ± 0.3 days, *and the halving time for the removal of non-clonogenic tumour cells as* 3.5 ± 0.7 days.

time after irradiation (Fig. 6, chart A). From the straight line, calculated by weighted regression (by weighting each point with the inverse square of its standard deviation) the doubling time of the number of clonogenic tumour cells was calculated to be 4.4 ± 0.3 days.

Immediately after irradiation, $52 \pm 14\%$ of the cells in the tumours are non-clonogenic tumour cells (Fig. 6, chart B). This number decreases exponentially with a halving time of 3.5 ± 0.7 days, as calculated by weighted regression using the data between Day 0 and Day 13. Since the number of non-clonogenic tumour cells is obtained as the difference of 2 nearly equal quantities, scatter is large. After Day 15, 10–20% of the cells [or a fraction increasing with $N_T(t)$] seem to be non-clonogenic tumour cells which mainly stems from the fact that the clonogenic fraction does not return to 100% of the controls but remains at about 80% [Fig. 3, curve $C_T(t)$]. The reason for this is not yet clear. It might be that some of the radiation-sterilized cells really remain in the irradiated tumours; but it is also possible that abortive divisions lead to a certain fraction of dead progeny cells or that the control level of clonogenic tumour cells decreases somewhat with age or size of the tumours.

DISCUSSION

The results of the present paper show that a variety of cell population kinetic changes occur in the R1H rhabdomyosarcoma after X-irradiation with 15 Gy. The most relevant observations are now discussed:

(1) *In irradiated tumours, the cell numbers and the ratio of tumour to host cells undergo drastic changes*

In unirradiated R1H tumours, 55% of the cells are tumour parenchymal cells (cf. Fig. 1A). Similar figures have been reported for some other transplantable tumours (e.g. 42–44% [19] or 52% [20]), but there are also several other tumours for which the proportion of tumour cells was found to be higher (e.g. 85–90% [6] or 85–95% [21]). The remaining proportions of cells in these tumours were attributed to host-derived cells.

The type and origin of what we call "host cells" have not been further characterized in the present study. However, it has been shown that, besides the fixed cellular elements of the supporting stroma such as fibroblasts and endothelial cells [22, 23], a number of blood-borne cell types contribute to the cellular composition of solid tumours (cf. [19] and the references cited there). This is supported by the observation that growth of FS6 fibrosarcomas was reduced when transplanted into whole-body irradiated mice [19, 20]. Concomitantly, these tumours showed a decrease in the proportion of macrophages. After the irradiated mice had been reconstituted by syngeneic marrow cells, tumour growth and cellular composition returned to nearly normal levels [19]. These findings imply that bone-marrow-derived blood-borne cells contribute measurably to tumour volume.

Following irradiation, the number of host cells present in the tumours increased by a factor of about 10 within 8 days (cf. Fig. 5). This effect is mainly attributed to an infiltration of irradiated tumours by blood-borne cells, rather than to a rapid proliferation of those present in the tumours. This interpretation is supported by the results of Stephens and coworkers [6] who reported that cell suspensions prepared from untreated Lewis lung carcinomas contained about 10% of host cells, mainly cells of the monocyte–macrophage series, the proportion of which increased shortly after 25 Gy of local irradiation, and for a few days comprised about 70–80% of the cells in the regenerating tumours. Since in our system, the host cells are considerably smaller than the tumour cells, this leads to the increase in cellular numerical density observed after irradiation (Fig. 4).

We have shown that the nuclear volume of tumour cells increased and reached a maximum by Day 10 after irradiation (cf. Fig. 2). Since this pathologic nuclear oedema should be taken as a morphological expression of the prelethal state of these cells, the decrease in the average nuclear volume observed after Day 10 does not mean that the cells return to normal size but more probably reflects the decrease in the proportion as well as the disintegration of the swollen cells. Similar effects have been reported to occur in the Walker carcinoma after X-irradiation [24] and in the B16 melanoma where the cell diameter reached its maximum 7–10 days after treatment with various chemotherapeutic drugs [7]. In part, these effects might account for the reduction in cell yield observed when tumours were treated with X-rays, neutrons or chemotherapy [3, 7, 25–27].

(2) *After irradiation, the surviving tumour cells repopulate the R1H tumours with a doubling time of about* 4 days.

From the data plotted in Fig. 6 (chart A),

the doubling time for the number of clonogenic tumour cells was determined by regression analysis to amount to 4.4 ± 0.3 days. Since in the unirradiated tumours, the initial volume doubling time is 3.6 days [10], repopulation of irradiated R1H tumours occurs at a rate that is comparable to the volume changes of unirradiated tumours. This result is at variance with the findings of earlier investigators [3, 5, 6] who reported that surviving cells repopulate irradiated tumours at a rate which is a factor of 2 or 3 larger than the rate of increase of cells in unirradiated tumours. This increase was partly ascribed to a shorter cell cycle time [5]. At present, it is not possible to say whether these discrepancies come from differences in the tumour systems used or from experimental sources such as determining repopulation rates from the fraction of clonogenic cells or from cell numbers derived from cell suspensions (see Discussion of the methods applied).

The data of Fig. 6(A) indicate that repopulation of R1H tumours begins immediately or within 2 days following irradiation. There might be a division delay of about 2 days, as indicated by Figs. 4 and 5. This is supported by our earlier findings that in R1H tumours irradiated with 4.25 Gy, accumulation of tumour cells in the G2 phase reached a maximum 15 hr after irradiation [28]; for the adenocarcinoma 284 irradiated with 6 or 12 Gy, a synchronization period of 24–48 hr has been reported [29]. Our data agree with the findings of Stephens *et al.* [6] who showed that in Lewis lung carcinomas irradiated with 15–35 Gy, repopulation began immediately or within 2 days; in contrast, the lag period reported for the R-1 rhabdomyosarcoma [3] was considerably longer than found in the present study.

(3) *After irradiation, the number of non-clonogenic tumour cells decreases exponentially with a halving time of about* 3.5 days

From the data plotted in Fig. 6 (chart B) the half-value time for the decrease of the number of non-clonogenic tumour cells was determined by regression analysis to amount to 3.5 ± 0.7 days. To our knowledge this represents the first direct measurement of the rate of removal on non-clonogenic tumour cells from an irradiated solid tumour.

There have been several previous attempts to determine radiation-induced cell loss in various animal tumours [2, 5, 30–32] which in most cases were analysed at the stage when the tumours had regrown to the volumes which were measured at the time of irradiation. The rate of cell loss was derived from the difference between the rate of cell production and the rate at which the tumour volume increased. But a number of assumptions implied in the ideal model of cell proliferation [8] no longer hold after irradiation (see also Conclusions) so that cell loss factors are of doubtful validity in irradiated tumours [2]. The method of measuring tumour cell loss rates *in situ* following radioactivity loss of ^{125}I-iododeoxyuridine [33] has been shown to be applicable to undisturbed tumours under certain restrictions [34, 35] and to serve as an indicator for the response of tumours to various treatments [36], but after irradiation there appears to be no correlation between cell loss factor and ^{125}IUdR loss [37]. Even more complicated is the problem of determining cell loss rate in human tumours; nevertheless, it has been reported that, during fractionated radiotherapy, the regression half-time of killed cells was 15 days [38] or 17 days [39].

The results of the present study cannot be compared with those of the papers cited above. The reasons are not only the problems associated with the methods applied or the fact that the experimental systems used differ in many respects (such as type and size of tumour, radiation dose, time interval between irradiation and measurements, etc.), but more importantly that we are dealing with different effects. Using the methods applied here, the disappearance of non-clonogenic but morphologically "intact" tumour cells from the tumour is measured so that cells that are lost into blood and lymphatic vessels are considered as well as cells that disintegrate within the tumour. The rate at which necrotic material is resorbed from the tumour does not affect our results, whereas cell loss factors and ^{125}IUdR loss are affected, especially in tumours where the development of necrosis exceeds resorption and emigration. In addition, the two latter methods are applicable only if there is no substantial infiltration of tumours by host cells. This effect does not play a role when the analysis is performed in terms of cell numbers.

As is evident from Fig. 6(B), the number of non-clonogenic tumour cells starts to decrease immediately after irradiation. There are no indications that a considerable fraction of these non-clonogenic cells divide a few times before they are eliminated. "Doomed cells" (i.e. cells that have lost the capacity for

unlimited proliferation but do not die instantaneously but may continue metabolizing and cycling for one or more post-treatment cycles) have been shown to exist in irradiated cell cultures by time lapse cinemicrography [40, 41] or replating the assayed cells after various time intervals [42, 43]. For various experimental tumours it has been suggested that, after irradiation, non-clonogenic cells might undergo several divisions before they die and disintegrate [3, 5, 44, 45] in order to explain why irradiated tumours may still continue to grow for a few days following exposure although the fraction of clonogenic tumour cells has been reduced by one or two orders of magnitude. This deduction is at variance with the results of the present study showing that post-irradiation growth of R1H tumours is not substantially affected by proliferation of doomed cells but is mainly due to an infiltration by host cells.

Discussion of the methods applied

The present study has been performed on a rather limited number of tumours, mainly because the determination of the numerical density of tumour cells is a very laborious procedure. For instance, each measured point of curve $D_T(t)$ in Fig. 3 required 4000 random points to be scored and the length of 300 linear intercepts to be measured. Although the amount of work involved in determining this parameter represents a real disadvantage for the application of our experimental procedure to large numbers of tumours, the knowledge of the numerical tumour cell density is absolutely necessary for describing the cell kinetic changes in irradiated tumours on the basis of cell numbers.

Many tumours, especially after irradiation, have large necrotic centers, making it impossible to interpret volume measurements in terms of cell numbers. It is not feasible to derive absolute cell numbers from cell suspensions since only 10–30% of the cells are released by trypsinization [7, 17], and this proportion changes with time interval after irradiation, as we have observed with the R1H tumour.

The importance of using cell numbers rather than cell ratios for cell kinetic analysis may be illustrated by the following considerations:

When the rate of repopulation was determined only from the fraction of clonogenic tumour cells (Fig. 3, curve $C_T(t)$; data for Day 0–8), a doubling-time of 2.5 ± 0.2 days was obtained. This value is considerably lower than that of 4.4 ± 0.3 days obtained from the analysis of the number of clonogenic tumour cells (Fig. 6A), indicating that repopulation data derived from the clonogenic fraction may lead to an overestimate of the rate at which tumours are repopulated after therapeutic treatment.

On the other hand, if the changes in the ratio of tumour to host cells are taken into consideration, the fraction of clonogenic tumour cells is obtained as the product of the clonogenic fraction and the proportion of tumour cells [Fig. 3, curves $C_T(t)$ and $P_T(t)$]. As becomes obvious from Fig. 3, the two curves compensate each other such that their product is constant from Day 0 to Day 10, thus leading to the conclusion that the fraction of clonogenic tumour cells remains fairly constant within the first 10 days after irradiation. This would be a considerable underestimation of the initial repopulation rate. The two examples given above strongly indicate that the kinetics of repopulation should not be determined from the proportion but rather from the number of clonogenic tumour cells per tumour. They also might, in part, explain some of the differences between our results and those reported for the original R-1 tumour [3, 5, 25] showing that after a lag period of several days repopulation occurred considerably faster than proliferation of tumour cells in unirradiated controls.

CONCLUSIONS

From the results presented and discussed so far, the following conclusions may be drawn:

(1) The kinetics of depopulation and repopulation occurring in irradiated tumours may be determined quantitatively if the number of tumour cells and of host cells per tumour as well as the clonogenic fraction of tumour cells are known.

(2) After irradiation, growth of R1H tumours largely depends on an increase in the number of host cells, mainly due to an infiltration by blood-borne cells which, by Day 8–10 after 15 Gy of local X-irradiation, comprise about 95% of the cells in the regenerating tumours.

(3) There are no indications that proliferation of doomed tumour cells contributes considerably to growth of R1H tumours after irradiation.

(4) The rate of clearance of radiation-sterilized cells cannot be assessed by conventional cell kinetic analysis since consider-

able infiltration of tumours by host cells occurs after irradiation which negates the basic assumptions implied in the underlying models.

Acknowledgements—We thank Professor G. W. Barendsen, Rijswijk, The Netherlands, for providing the R-1 tumour and for many stimulating discussions. We thank Sylvia Bornkeβel, Heinke Kölling, Susanne Mücke, Maria Omniczynski and Claudia Ramm for skilful technical assistance. This work was supported by the Bundesministerium für Forschung und Technologie (Förderungskennzeichen: 01 ZL 058).

REFERENCES

1. T. C. Stephens and G. G. Steel, Regeneration of tumours after cytotoxic treatment. In *Radiation Biology in Cancer Research.* (Edited by R. E. Meyn and H. R. Withers) p. 385. Raven Press, New York (1980).
2. J. Denekamp and R. H. Thomlinson, The cell proliferation kinetics of four experimental tumors after acute X-irradiation. *Cancer Res.* **31,** 1279 (1971).
3. G. W. Barendsen and J. J. Broerse, Experimental radiotherapy of a rat rhabdomyosarcoma with 15 MeV neutrons and 300 kV X-rays. I. Effects of single exposures. *Europ. J. Cancer* **5,** 373 (1969).
4. N. J. McNally, A comparison of the effects of radiation on tumour growth delay and cell survival. The effect of oxygen. *Brit. J. Radiol.* **46,** 450 (1973).
5. A. F. Hermens and G. W. Barendsen, Changes of cell proliferation characteristics in a rat rhabdomyosarcoma before and after X-irradiation. *Europ. J. Cancer* **5,** 173 (1969).
6. T. C. Stephens, G. A. Currie and J. H. Peacock, Repopulation of γ-irradiated Lewis lung carcinoma by malignant cells and host macrophage progenitors. *Brit. J. Cancer* **38,** 573 (1978).
7. T. C. Stephens and J. H. Peacock, Cell yield and cell survival following chemotherapy of the B16 melanoma. *Brit. J. Cancer* **38,** 591 (1978).
8. G. G. Steel, *Growth Kinetics of Tumours.* Clarendon Press, Oxford (1977).
9. H. B. Kal, Proliferation behaviour of P and Q cells in a rat rhabdomyosarcoma after irradiation as determined by DNA measurements. *Europ. J. Cancer* **9,** 753 (1973).
10. H. Jung, H.-P. Beck, I. Brammer and F. Zywietz, Factors contributing to tumour growth after irradiation. *Brit. J. Cancer* **41,** Suppl. IV, 226 (1980).
11. H.-P. Beck, Evaluation of flow cytometric data of human tumours. Correction procedures for background and cell aggregations. *Cell Tiss. Kinet.* **13,** 173 (1980).
12. E. R. Weibel, *Stereological Methods.* Vol. 1. *Practical Methods for Biological Morphometry.* Academic Press, New York (1979).
13. H. W. Chalkley, Method for the quantitative morphologic analysis of tissues. *J. nat. Cancer Inst.* **4,** 47 (1943).
14. E. R. Weibel, Principles and methods for the morphometric study of the lung and other organs. *Lab. Invest.* **12,** 131 (1963).
15. W. G. Cochran, *Sampling Techniques.* Wiley, New York (1977).
16. H. E. Exner, Analysis of grain- and particle-size distributions in metallic materials. *Int. metal. Rev.* **17,** 25 (1972).
17. H. S. Reinhold, A cell dispersion technique for use in quantitative transplantation studies with solid tumours. *Europ. J. Cancer* **1,** 67 (1965).
18. H. Jung, Eine einfache Anordnung zum Auszählen von Zellkolonien. *Leitz-Mitt. Wiss. Techn.* **7,** 102 (1978).
19. R. Evans, Macrophage requirement for growth of a murine fibrosarcoma, *Brit. J. Cancer* **37,** 1086 (1978).
20. R. Evans, Effect of X-irradiation on host-cell infiltration and growth of a murine fibrosarcoma. *Brit. J. Cancer* **35,** 557 (1977).
21. D. J. Grdina, S. Linde and K. Mason, Response of selected tumour cell populations separated from a fibrosarcoma following irradiation *in situ* with fast neutrons. *Brit. J. Radiol.* **51,** 291 (1978).
22. I. F. Tannock, Population kinetics of carcinoma cells, capillary endothelial cells, and fibroblasts in a transplanted mouse mammary tumor. *Cancer Res.* **30,** 2470 (1970).
23. I. Brammer, F. Zywietz and H. Jung, Changes of histological and proliferative indices in the Walker carcinoma with tumour size and distance from blood vessel. *Europ. J. Cancer* **15,** 1329 (1979).

24. W. A. LINDEN, A. CASTANO Y ALMENDRAL and R. FRISCHKORN, Karyometrische Untersuchungen am Walker-Karzinom nach Einzeitbestrahlung mit verschiedenen Dosen ^{60}Co-Gamma- bzw. 200 kV-Röntgenstrahlung. *Strahlentherapie* **123,** 1 (1964).
25. G. W. BARENDSEN and J. J. BROERSE, Experimental radiotherapy of a rat rhabdomyosarcoma with 15 MeV neutrons and 300 kV X-rays. II. Effects of fractionated treatments, applied five times a week for several weeks. *Europ. J. Cancer* **6,** 89 (1970).
26. C. J. KOVACS, H. A. HOPKINS, M. J. EVANS and W. B. LOONEY, Changes in cellularity induced by radiation in a solid tumour. *Int. J. Radiat. Biol.* **30,** 101 (1976).
27. T. C. STEPHENS, J. H. PEACOCK and G. G. STEEL, Cell survival in B16 melanoma after treatment with combinations of cytotoxic agents: Lack of potentiation. *Brit. J. Cancer* **36,** 84 (1977).
28. F. ZYWIETZ and H. JUNG, Partial synchronization of three solid animal tumours by X-rays. *Europ. J. Cancer* **16,** 1381 (1980).
29. L. V. SZCZEPANSKI and K. R. TROTT, Post-irradiation proliferation kinetics of a serially transplanted murine adenocarcinoma. *Brit. J. Radiol.* **48,** 200 (1975).
30. J. S. R. NELSON, R. E. CARPENTER and D. DURBORAW, Mechanisms underlying reduced growth rate in C3HBA mammary adenocarcinomas recurring after single doses of X-rays or fast neutrons. *Cancer Res.* **36,** 524 (1976).
31. A. F. HERMENS and G. W. BARENDSEN, The importance of proliferation kinetics and clonogenicity of tumor cells for volume responses of experimental tumors after irradiation. In *Radiation Research: Biomedical, Chemical and Physical Perspectives*, (Edited by O. F. Nygaard, H. I. Adler and W. K. Sinclair) p. 834. Academic Press, New York (1975).
32. M. POTMESIL, D. LUDWIG and A. GOLDFEDER, Cell kinetics of irradiated experimental tumors: Relationship between the proliferating and the non-proliferating pool. *Cell Tiss. Kinet.* **8,** 369 (1975).
33. W. PORSCHEN and L. FEINENDEGEN, *In-vivo*- Bestimmung der Zellverlustrate bei Experimentaltumoren mit markiertem Joddeoxyuridin. *Strahlentherapie* **137,** 718 (1969).
34. A. C. BEGG, Cell loss from several types of solid murine tumours: comparison of ^{125}I-iododeoxyuridine and tritiated thymydine methods. *Cell Tiss. Kinet.* **10,** 409 (1977).
35. L. A. DETHLEFSEN, J. SORENSON and J. SNIVELY, Cell loss from three established lines of the C3H mouse mammary tumor: a comparison of the ^{125}I-UdR and the ^{3}H-TdR-autoradiographic methods. *Cell Tiss. Kinet.* **10,** 447 (1977).
36. W. PORSCHEN, J. GARTZEN, K. GEWEHR, H. MÜHLENSIEPEN, H.-J. WEBER and L. E. FEINENDEGEN, *In vivo* assay of the radiation sensitivity of hypoxic tumour cells; influence of γ-rays, cyclotron neutrons, misonidazole, hyperthermia and mixed modalities. *Brit. J. Cancer* **37,** Suppl. III, 194 (1978).
37. A. J. FRANKO, R. F. KALLMAN, D. RAPACHIETTA and S. D. KELLEY, ^{125}IUdR loss as a measure of tumour cell loss: influence of reutilization and influx of labelled host cells. *Brit. J. Cancer* **41,** Suppl. IV, 69 (1980).
38. Y. OKUMURA, T. UEDA, T. MORI and T. KITABATAKE, Kinetic analysis of tumour regression during the course of radiotherapy. *Strahlentherapie* **153,** 35 (1977).
39. J. DUTREIX and A. WAMBERSIE, Radiobiologic data obtained from clinical observation of the regression of epithelioma of the tonsil under different fractionation regimes. *Amer. J. Roentgenol.* **108,** 37 (1970).
40. C. HURWITZ and L. J. TOLMACH, Time lapse cinemicrographic studies of X-irradiated HeLa S3 cells. I. Cell progression and cell disintegration. *Biophys. J.* **9,** 607 (1969).
41. K.-R. TROTT, Strahlenwirkung auf die Vermehrung von Säugetierzellen. In *Handbuch der Medizinischen Radiologie*, Vol. 3, part 3. p. 43. Springer, Berlin (1972).
42. I. R. HEYDER and W. POHLIT, Proliferation of lethally damaged mammalian cells irradiated by fast electrons, fast neutrons, α particles, and pions. *Radiat. Res.* **82,** 352 (1980).

43. H. JUNG, Post-irradiation growth kinetics of surviving and non-surviving cells. *Radiat. environm. Biophys.* (submitted for publication).
44. S. ROCKWELL, E. FRINDEL, A. J. VALLERON and M. TUBIANA, Cell proliferation in EMT6 tumors treated with single doses of X-rays or hydroxyurea. I. Experimental results. *Cell Tiss. Kinet.* **11,** 279 (1978).
45. A. F. HERMENS and G. W. BARENDSEN, The proliferative status and clonogenic capacity of tumour cells in a transplantable rhabdomyosarcoma of the rat before and after irradiation with 800 rad of X-rays. *Cell Tiss. Kinet.* **11,** 83 (1978).