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Partial Synchronization of Three Solid Animal Tumours by X-Rays

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Abstract—*Three different solid tumours (rhabdomyosarcoma R1H, Walker-256 carcinoma, adenocarcinoma E0771) were investigated with respect to partial synchronization using the technique of flow cytometry. Tumours at different stages of growth were locally irradiated with* 425 rad *of* 200 kVp *X-rays. After staining with ethidium bromide and mithramycin, the DNA distribution patterns of the cells were recorded using a flow cytometer Phywe ICP* 11. *A computer analysis of the DNA histograms was performed yielding the percentage of cells in the G_1-, S-, and G_2+M-phase of the cell cycle. Local irradiation of the tumours leads to an increase of cells in the G_2+M-phase, which amounts to* 23.8 *and* 21.7% *in the case of the two carcinomas (at* day 7*) but only to* 9.7% *for the rhabdomyosarcoma (at* day 19*). In the carcinomas, when irradiated at* day 14 *after transplantation, accumulation is about half compared to* day 7. *From the results presented it is concluded that the positive clinical results after 'synchronization radiotherapy' reported so far probably cannot be attributed to an accumulation of tumour cells in a radiosensitive phase of the cell cycle.*

INTRODUCTION

'SYNCHRONIZATION radiotherapy' has been applied in clinical treatment of cancer for about 10 years (cf. [1] and the reviews [2–5]). Nevertheless, in all these years, it has not been possible to prove that the positive clinical results reported (cf. [6, 7]) are due to an accumulation of tumour cells in an especially radiosensitive phase of the cell cycle. On the contrary, the cell kinetic basis of this therapeutic modality, although appearing highly plausible, has been subjected to severe criticism [8, 9], since relatively early on.

Numerous radiobiological publications, as well as data from our laboratory [10], show that mammalian cells are most radiosensitive in early G_2-phase. Accumulation of cells in G_2-phase can be achieved by a variety of different methods. Earlier experiments, using several cytostatic drugs [11] or X-rays [12], showed that in cell cultures 60–80% of the cells treated may be accumulated in G_2+M-phase, whereas in animal tumours accumulation was much less [13, 14]. The advantage of using X-rays appears to be two-fold. On one hand, the extent of accumulation achieved *in vivo* by local irradiation [13] was found to be substantially higher than that caused by non-toxic concentrations of daunomycin [15] or hydroxyurea [14, 16]. On the other hand, the synchronizing effects of X-rays are mainly restricted to the tumour itself without much affecting the tumour-bearing host as is the case with most chemical agents. Since the radiobiological and cell kinetic basis of 'synchronization radiotherapy' is still under discussion, we performed a series of experiments on three solid animal tumours, aiming at a detailed description of the extent and the kinetics of accumulation of cells in G_2+M-phase caused by local X-irradiation of the tumours.

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MATERIALS AND METHODS

In order to investigate to what extent the accumulation achieved depends on the growth characteristics of the tumours treated, the experiments were performed using a rhabdomyosarcoma R1H [17] of the rat which grows rather slowly, and two fast-growing tumours, the Walker-carcinoma 256 of the rat and the adenocarcinoma E0771 of the mouse. The R1H and E0771 tumours do not show any detectable immunogenicity [18, 19] whereas the Walker-256 is highly immunogenic [20]. Their patho-histological classification is presented in Table 1.

*Table 1. Patho-histological classification of the solid animal tumours used**

Tumour system	Year and site of origin	Pathology
Rhabdomyosarcoma R1H (rat)	1962: jaw musculature	Solid tumour with foci of necrosis, but enclosed by a fibrous capsule
Walker-256 carcinoma (rat)	1928: mammary gland	Solid tumour with foci of necrosis, with infiltration of the contiguous soft tissues
Adenocarcinoma E0771 (mouse)	1940: mammary gland	Solid (medullary) tumour without glandlike differentiations, with foci of necrosis, with destruction and infiltration of the adjoining soft tissues

*Performed by Prof. Dr. H. F. Otto, Institute of Pathology, University of Hamburg.

The rhabdomyosarcoma R1H (H = Hamburg) was transplanted by inoculating a piece of tumour tissue of about 1 mm^3 s.c. into the right flank of male WAG/Rij albino rats (210 ± 10 g). For transplantation of the Walker-256 carcinoma, 0.05 ml of a tumour homogenate (5×10^6 cells) were injected s.c. into the thigh of young male BD1 rats weighing 220 ± 10 g. The adenocarcinoma E0771 was transplanted on male (23 ± 2 g) C57B1/6j-mice by injecting 0.3 ml of a tumour homogenate (1.8×10^5 cells) into the muscle of the hind leg. All animals were kept under a 12-hr light, 12-hr dark regimen and provided with food and water *ad libitum*.

Tumour volumes were determined by measuring the tumours 3–5 times per week in three perpendicular dimensions, using vernier calipers. From the growth curves the volume doubling times were obtained. In the earlier stage, the carcinomas have doubling times of 1.0 and 2.1 days, respectively. The growth rate decreases with increasing tumour age. The rhabdomyosarcoma R1H grows more slowly with a volume doubling time of 3.6 days [17]. The age and volume of tumours used for the experiments are indicated in Table 2.

The tumours were locally irradiated with 425 rad of 200 kVp X-rays (0.5 mm Cu filtering), the dose rate being 100 rad/min. For exposure, the rats were anaesthesized with sodium pentobarbitone (Nembutal®), injected i.p. at 60 mg/kg body wt 10 min before irradiation. The mice were irradiated in lucite boxes without anaesthetic.

The degree of partial synchronization after irradiation was determined by flow cytometry (FCM). From the middle of the tumour, a slice of tumour tissue was excised and minced, and a cell suspension was prepared according to the method described by Roters *et al.* [21]. The DNA fluorescence distribution was recorded with a flow cytophotometer ICP 11 (Phywe AG, Göttingen, W. Germany) on line with a Wang 2200B computer. From the DNA

Table 2. Accumulation of tumour cells in $G_2 + M$ phase caused in tumours of different ages by irradiation with 425 rad of X-rays

Tumour (time after transplantation)	Tumour volume (cm^3)	Tumour volume doubling time (t_d)	Percentage of cells in $G_2 + M$ phase $f(G_2 + M)$, (%)		Time for maximum accumulation Δt (hr)	Rate of accumulation $\Delta f(G_2 + M)/\Delta t$ (%/hr)
			Before irradiation	After irradiation		
Rhabdomyosarcoma R1H						
(day 19)	1.6 ± 0.2	3.6	8.8 ± 1.7	18.5 ± 1.3	15	0.65
Walker-256 carcinoma						
(day 7)	2.5 ± 0.5	1.0	10.6 ± 2.2	34.4 ± 3.8	10	2.38
(day 14)	14.0 ± 3.1	7.2	11.1 ± 1.7	27.3 ± 3.1	12	1.35
Adenocarcinoma E0771						
(day 7)	0.7 ± 0.1	2.1	14.7 ± 2.6	36.4 ± 4.4	10	2.17
(day 14)	3.4 ± 0.5	4.3	11.1 ± 0.7	22.5 ± 3.1	10	1.14

distribution patterns derived from about 60,000 cells, the fraction of cells in G_1-, S- and G_2+M-phase were calculated by a computer program developed by Baisch *et al.* [22]. For each series of experiments about 50 tumours were investigated.

RESULTS

Figure 1 shows, as an example, a series of DNA histograms obtained at various times after irradiation of Walker carcinomas with 425 rad. The DNA histograms were cut off left from the G_1-peak by a preset threshold, thus eliminating leucocytes and cell debris. On each chart, the percentages of cells in G_1-, S- and G_2+M are given. After irradiation, the fraction of G_2+M-cells increases with time, reaches a maximum of 35.6% after 10 hr and then decreases again. This indicates that the cells are accumulated in the G_2+M-phase by irradiation and that this block is 'reversible' (cf. Discussion) after about 10 hr. In addition, the fraction of S-cells decreases from 39.9 to 20.5% in the course of 10 hr demonstrating another block to exist at the G_1/S-transition as we have previously shown for cells in culture [12] and animal tumours [14].

Similar series of histograms were recorded for all irradiated tumours over a period of 24 hr after exposure and the percentages of cells in the phases G_1, S and G_2+M were calculated as described above. The data are shown in Figs. 2, 3 and 4 where each point represents the mean ($\pm$S.D.) of 5–6 tumours studied.

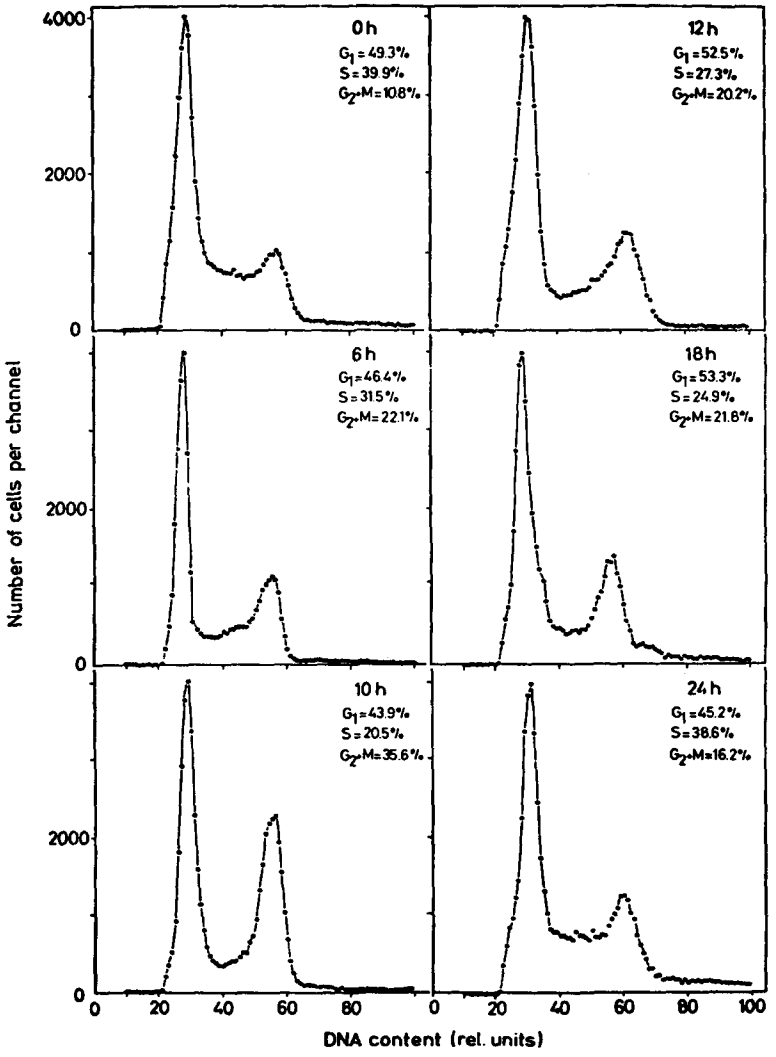

Fig. 1. DNA fluorescence distribution patterns of Walker-256 carcinomas (day 7) *recorded at various times after irradiation with* 425 rad *X-rays. The percentage of cells in the phases G_1, S. and G_2+M are indicated in each chart.*

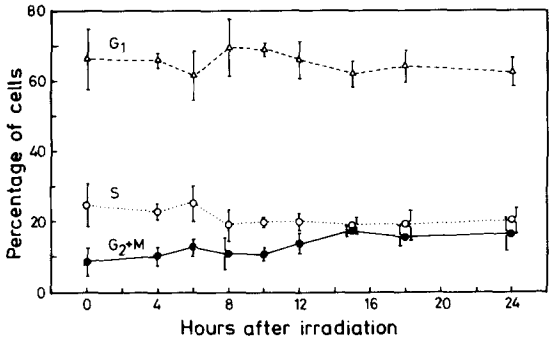

Fig. 2. Percentage of cells of the rhabdomyosarcoma R1H of the rat (day 19 *after transplantation*) *in the phases G_1, S and G_2+M of the cell cycle recorded at various time intervals after irradiation with* 425 rad *of X-rays.*

Figure 2 shows the cell kinetic changes occurring in irradiated rhabdomyosarcomas. In the controls, $66.3 \pm 3.6\%$ of the cells are in G_1-phase (since the peak at 2c DNA content contains also the G_0-cells, it should be named G_0+G_1), $24.9 \pm 2.7\%$ in S-phase and $8.8 \pm 1.7\%$ in G_2+M-phase. The G_2+M content increases with time after irradiation and reaches a maximum of $18.5 \pm 1.3\%$ 15 hr after exposure with 425 rad.

Figure 3 shows the data for irradiated Walker carcinomas. In tumours irradiated at day 7 after transplantation (3A), the fraction of cells in G_2+M increases from 10.6% in the asynchronous control population up to 34.4%.

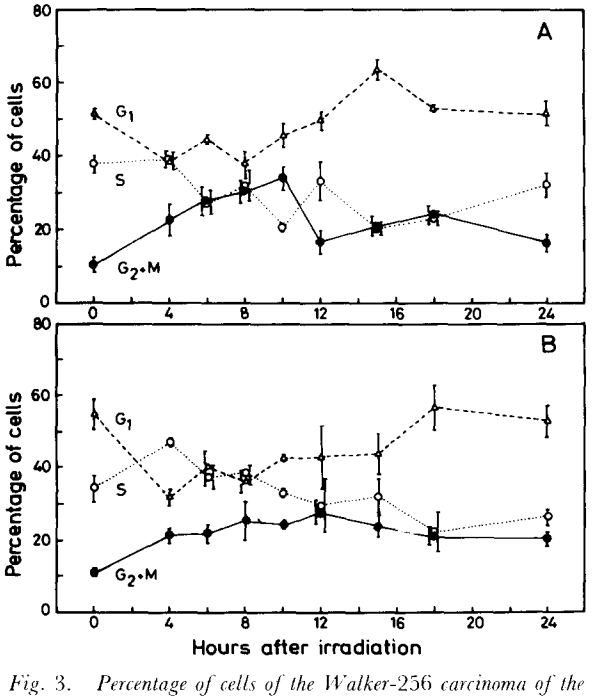

Fig. 3. Percentage of cells of the Walker-256 carcinoma of the rat in the phases G_1, S and G_2+M of the cell cycle recorded at various time intervals after irradiation with 425 rad *of X-rays.* (*A*) *Tumours at* day 7 *after transplantation.* (*B*) *Tumours at* day 14 *after transplantation.*

The increase is about linear, maximum accumulation is reached 10 hr after irradiation. In the following hours, the fraction of G_2+M-cells decreases rapidly and that of G_1-cells increases showing that the blockage is reversible and that the cells are leaving the G_2-phase partly synchronized and enter G_1. For 14-day-old Walker carcinomas (Fig. 3B), accumulation occurs more slowly as compared to 7-day-old tumours. A maximum of $27.3 \pm 3.1\%$ cells in G_2+M is reached 12 hr after irradiation.

Figure 4 shows the results obtained for adenocarcinomas of the mouse. On the whole, the data are comparable to those plotted in

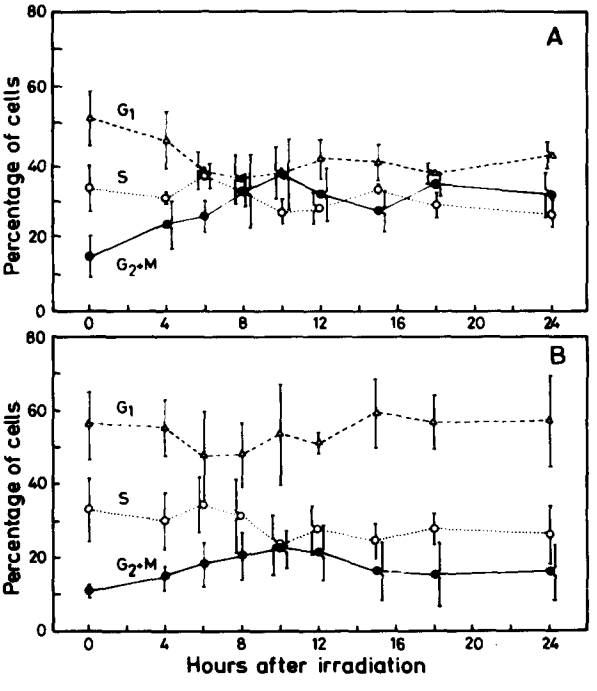

Fig. 4. Percentage of cells of the adenocarcinoma E0771 of the mouse in the phases G_1, S and G_2+M of the cell cycle recorded at various time intervals after irradiation with 425 rad *of X-rays.* (*A*) *Tumours at* day 7 *after transplantation.* (*B*) *Tumours at* day 14 *after transplantation.*

Fig. 3. In 7-day-old tumours, the portion of G_2+M-cells increases from 14.7 to 36.4%, whereas in the older tumours the increase is from 11.1 to 22.5%. In either tumour, maximum accumulation occurs 10 hr after irradiation.

Table 2 gives a summary of the results obtained for the three tumours. In all cases, an accumulation of tumour cells in the G_2+M-phase was observed but the amount of accumulation was different. In the smaller tumours, a dose of 425 rad enhanced the fraction of G_2+M-cells by 9.7% in the rhabdomyosarcoma, by 23.8% in the Walker car-

cinoma and by 21.7% in the adenocarcinoma. From these figures and the time interval necessary to reach the maximum, the rate of accumulation has been obtained (Table 2, last column). For the R1H tumours, the rate of G_2+M accumulation amounts to 0.65%/hr whereas for the two carcinomas higher but comparable values are found: 2.17 and 2.38%/hr for day 7, and 1.14 and 1.35%/hr for day 14, respectively.

DISCUSSION

The accumulation of cells in G_2+M-phase reported is caused by a block in G_2 but not in M. This becomes obvious from the fact that the mitotic index decreases with time after irradiation as we and many others have shown for cell cultures [11, 12] and animal tumours [13, 14]. In order to show whether the different accumulation rates measured for the rhabdomyosarcoma and the two carcinomas (cf. Table 2) may be correlated with any cell kinetic properties of the tumours investigated, our results and several other parameters determined in our laboratory are compiled in Table 3. This comparison was possible only for the smaller tumours since for the larger ones the corresponding data were not available. Since the so-called 'G_1' peak of flow cytometric (FCM) histograms contains proliferating and non-proliferating cells, the fraction of non-proliferating cells (1-GF) was subtracted from the 2c peak to determine the fraction (f_x) of cells in G_0 and in G_1. Knowing the cell cycle time (t_c) and the growth fraction (GF), the length of the various phases may be calculated from Steel's formula [26]

$$t_x = t_c \cdot \frac{\log_e [1+(\alpha-1)\cdot f_x]}{\log_e \alpha}$$

where t_x is the length of the last phase(s) in the cycle, f_x is the fraction of cells in the last phase(s) and $\alpha = GF+1$. The duration of the phases $S+G_2+M$ correlates well with the time Δt required for maximum accumulation (cf. Table 2) indicating that the maximum is reached when the cells that have been irradiated in S and G_2 have arrived at the radiation-induced block in G_2-phase. Since another block is induced at the G_1/S-transition, after the time of $S+G_2$ a reduced number of cells migrate through S-phase thus leading to the observed decrease in the fraction of cells in G_2+M. Consequently, this decrease in G_2+M-cells does not mean that the G_2-block is reversible after 10–15 hr, but rather is a measure of the time interval by which the two blocks are spaced in the cell cycle. This conclusion is supported by our earlier findings [13] showing that in the Walker carcinoma, the accumulation of cells in the G_2-phase levels off at higher doses. The maximum of 48% G_2+M-cells measured after 900 rad agrees well with the corresponding content of cells in the phases S and G_2+M (Table 3), indicating that all cells between G_1/S and G_2 are accumulated at the G_2-

Table 3. Comparison of cell kinetic parameters of three animal tumours with accumulation in G_2+M-phase caused by irradiation with 425 rad X-rays

Tumours	Growth parameters	Percentage of cells in phases [duration of phases (hr)]				Accumulation rate, $f(G_2+M)/\Delta t$ (%/hr)		
		G_0	G_1	S	G_2+M	Expected‖	Measured	Ratio
Rhabdomyosarcoma R1H (day 19)	$t_c=18$ hr* $t_s=10$ hr* $GF=0.45$†	55	11.3	24.9 (10.0)	8.8 (4.1)	2.49	0.65	0.26
Walker-256 carcinoma (day 7)	$t_c=18$ hr‡ $GF=0.93$‡	7	44.4 (7.1)	38.0 (8.1)	10.6 (2.8)	4.69	2.38	0.51
Adenocarcinoma E0771 (day 7)	$t_c=19$ hr§ $GF=0.9$§	10	41.9 (7.4)	33.4 (7.6)	14.7 (4.0)	4.39	2.17	0.49

*PLM-data from Brammer (unpublished).
†Estimated from t_c, t_s and FCM data.
‡PLM-data from Erbe *et al.* [23] and Brammer *et al.* [24].
§PLM-data from König [25].
‖Obtained from percentage of cells in S phase divided by length of S phase, yielding the maximum accumulation rate to be expected if all S-cells are blocked in G_2.

block. In a similar way, the data of Raju *et al.* [27] may be interpreted to show that even after 3000 rad, the G_2+M content measured for the KHT-sarcoma of the mouse did not exceed 50%.

From these arguments, we may conclude that the block induced by lower doses, as used in the present study, cannot accumulate all the arriving cells, but that there is some 'leakage' through this G_2 block. A rough estimate of the 'leakage' rate may be obtained in the following way: In the rhabdomyosarcoma 24.9% of all cells (including G_0) are found in S-phase which has a duration of 10 hr. Thus, if all arriving cells are blocked in G_2, the maximum accumulation rate to be expected is 2.49%/hr (Table 3). The ratio of the accumulation rate measured and of the expected value (Table 3, last column) may be regarded as a measure for the efficiency of accumulation by the radiation-induced G_2-block. Hence, in the irradiated rhabdomyosarcoma about 25% of the arriving cells are blocked in G_2, whereas in the two carcinomas this ratio is about 50%. These figures should be regarded as lower limits, since there are some experimental data from ^{3}H-incorporation studies [28] as well as from FCM measurements [29] showing that progression through S-phase is slowed down by irradiation, which might lower the maximally achievable accumulation rate and thus slightly increase the efficiency of the block.

Not much can be said about the efficiency of the G_1/S-block in comparison to that in G_2. In the rhabdomyosarcoma, the G_2+M content, after reaching the maximum at 15 hr, decreases slowly with time (Fig. 2), indicating a high 'leakage' through the G_1/S-block. On the other hand, in the Walker carcinoma the decrease after 10 hr is more pronounced (Fig. 3), indicating a stronger block to exist at the G_1/S transition as compared to R1H. This might be regarded as an indication that the radiation-induced blocks at G_1/S and in G_2 are of comparable strength.

The validity of our conclusions cannot be tested since data from the literature are not available. Although the G_2-block of division after irradiation is a well-documented effect for cell cultures (cf. [30]) as well as for several *in vivo* systems (cf. references given by Rockwell *et al.* [31]), only a few publications give quantitative figures for radiation-induced G_2-block in solid animal tumours investigated by means of flow cytometry [14, 27, 32]. But, data of cell cycle times and growth fraction of the tumours used in the FCM studies cited are not known.

As far as the clinical applicability of 'synchronization radiotherapy' is concerned, our data allow to draw the following conclusions: Tubiana and Malaise [33, 34] have shown by analyzing the cell kinetic data from 257 patients, that the growth fraction of adenocarcinomas, mesenchymal sarcomas and squamous cell carcinomas range from 6 to 25%. Since, in addition, the cell cycle times of human tumours are generally longer than those of animal tumours [34–36], the G_2+M accumulation that can be achieved in human tumours should be much below the values reported in this study. This conclusion is supported by the findings of Wannenmacher *et al.* [37] who observed an average increase in G_2+M fraction by about 4.4% after treating 51 patients with 5-fluorouracil or bleomycin. For these reasons it is concluded that it is not possible to accumulate a sufficiently large portion of tumour cells in G_2+M-phase as to explain the positive clinical results reported so far on the basis of an enhanced radiosensitivity due to cell kinetic changes.

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